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A microbiological approach to improve the performance of single-chamber bioelectrochemical systems

- PhD Thesis -

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Dr. Juan Antonio Baeza Labat

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Ai miei genitori e a Petà

Al mio Grande Sud

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Abstract

The need of renewable energy and the constant threat of global warming have motivated the development of emerging sustainable technologies. Among them, bioelectrochemical systems envisage a future where energy and other added value products, such as hydrogen, can be obtained from organic waste streams. Thus, bioelectrochemical systems fit perfectly in the new paradigm with respect to wastes, which should be valorized rather than treated. Bioelectrochemical systems, also known with the name of microbial electrochemical systems (MXCs), combine the electrochemistry with the metabolism of a particular group of microorganisms called exoelectrogens or anode respiring bacteria (ARB).

The need of understanding the metabolic activity of ARB and their optimal growth conditions are of high importance to ensure the maximal performance of MXCs. In this frame, this thesis aimed to develop, to study and to upgrade the microbial community of MXCs in view of increasing their performance.

For this purpose, ribosomal 16S gene targeted metagenomics study and quantitative real-time PCR (qPCR) techniques were implemented in this thesis. qPCR curves were built to study the evolution of ARB and methanogen microbial communities. Further, 454 pyrosequencing analysis were conducted in order to deepen the composition of microbial electrodes communities.

Different experimental setups were designed to study operational conditions under which ARB could outcompete other microorganisms that can undermine MXCs performance, such as methanogens and homoacetogenic bacteria. The first step was the ARB enrichment at different external resistances in Microbial Fuel Cells (MFCs). This study showed that the anode inoculated under low external resistance (12 Ω) in MFC mode showed better performance in the posterior Microbial Electrolysis Cell (MEC) mode (i.e. gave higher current intensity and showed higher H_2 production rate) than other MFCs inoculated under higher resistances (220 and 1000 Ω). Moreover, qPCR confirmed that the use of a low external resistance provides an MFC anodic biofilm with the highest content of *Geobacter* (the most usual ARB).

A long term study of the most common methanogens chemical inhibitor: 2-bromoethanesulfonate (BES) was performed in two steps to underline its limitations. A long term acetate-fed MXCs showed BES ineffectiveness caused by *Archaea* (hydrogen-oxidizing genus *Methanobrevibacter*, of the *Methanobacteriales* order) resistance to high BES concentration (up to 200 mM) in MEC. In addition, BES degradation was demonstrated in MFC. Moreover, at higher BES concentration (200 mM), methanogenesis activity decreased but resulted in an increase of H_2 recycling by homoacetogenesis due to the favorable conditions for these microorganisms.

A MEC inoculated with *Geoalkalibacter ferrihydriticus* pure culture at high pH revealed high current intensity production (up to 10 $A \cdot m^{-2}$). These results suggested the possibility to use

alkaline conditions with the objective to improve MXCs performance by creating a more selective environment. Then, a high pH community was selected from anaerobic sludge using alkaline medium and an online pH control. High performances were obtained in both MFC and MEC (around 50 $mA \cdot m^{-2}$ of current density and 2.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). *Alkalibacter* genus was highly detected in an alkaline MFC (37% of the bacterial community) and it was identified as a potential ARB. The presence of *Geoalkalibacter* genus was confirmed at high pH (9.3) conditions and especially in MEC (43 %).

Finally, a successful strategy was developed with the purpose of obtaining a cheese-whey fermentative-ARB syntrophic community. Cheese-whey was fermented to acetate mainly by lactic acid bacteria (*Enterococcus* genus was 22% of the total bacterial community) and other fermenting bacteria as *Sphaerochaeta* and *Dysgonomonas* genera. Exoelectrogenic activity was performed by *Geobacter* sp. (37%), that used the acetate as electron donor. This microbial community was able to degrade cheese whey to produce directly energy or H_2 ($0.6\ L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). Moreover, cheese whey MXCs demonstrated the intrinsic ability to inhibit methanogenic activity without using other external inhibition strategies.

Resumen

La necesidad de energía renovable y la constante amenaza del calentamiento global han motivado el desarrollo de nuevas tecnologías más sostenibles que las actuales. Entre ellas se encuentran los sistemas bioelectroquímicos, que pueden permitir un futuro donde la energía y otros productos de valor añadido, como el hidrógeno, se logren obtener de nuestros residuos orgánicos. De este modo, dichos sistemas encajan perfectamente en el nuevo paradigma actual en cuanto a los residuos, que deben ser valorizados además de tratados. Los sistemas bioelectroquímicos, también conocidos con el nombre de celdas electroquímicas microbianas (Microbial Electrochemical Cells, MXCs), combinan la electroquímica con el metabolismo de un grupo particular de microorganismos llamados exoelectrógenos o bacterias que respiran gracias a un ánodo (Anode Respiring Bacteria, ARB).

Entender la actividad metabólica de los ARB y sus condiciones óptimas de crecimiento son de gran importancia para garantizar el máximo rendimiento de las MXCs. Por este motivo, la presente tesis estudia los sistemas bioelectroquímicos y tiene como objetivo desarrollar, estudiar y mejorar la comunidad microbiana de las MXCs para aumentar su rendimiento.

Se han aplicado técnicas moleculares avanzadas para el estudio de las poblaciones microbianas como por ejemplo el estudio metagenómico del gen 16S ribosomal, o la PCR en tiempo real (qPCR). Se han construido curvas qPCR para estudiar la evolución de los ARB y de las diferentes comunidades microbianas metanógenas existentes en los sistemas bajo estudio. Además, se han llevado a cabo experimentos con pirosecuenciación 454 para profundizar en la composición de las comunidades microbianas que crecen en la superficie de los electrodos.

Se han diseñado diferentes montajes experimentales para estudiar las condiciones de operación que favorecen a los ARB frente a otros microorganismos que disminuyen la eficiencia de las MXCs, como son los metanógenos o las bacterias homoacetógenas. El primer paso ha sido el enriquecimiento de ARB bajo diferentes resistencias externas en celdas de combustible microbianas (Microbial Fuel Cells, MFC). Este estudio ha demostrado que el ánodo inoculado en una MFC bajo una resistencia externa baja (12 Ω) tiene un mejor rendimiento cuando es trasladado a una celda de electrólisis microbiana (Microbial Electrolysis Cell, MEC); es decir, proporciona mayor intensidad y mayor tasa de producción de H_2 que otras MFC inoculadas con resistencias externas superiores (220 y 1000 Ω). Por otro lado, los análisis de qPCR han confirmado que el uso de una resistencia externa baja en MFC comporta la formación de una biopelícula anódica con mayor contenido de *Geobacter* (el ARB más habitual).

También se ha llevado a cabo un estudio en dos etapas para conocer las limitaciones del uso a largo plazo del inhibidor más común de la metanogénesis: el 2-bromoetanosulfonato (BES). En MFCs alimentadas con acetato, se ha observado como el BES se demostraba ineficaz a largo plazo, ya que no puede evitar el crecimiento de *Archaea* (concretamente del género que usa

hidrógeno como donador de electrones, *Methanobrevibacter*, de la orden *Methanobacteriales*). Incluso a niveles muy elevados de BES (hasta 200 mM) se ha observado producción de metano, lo que parece indicar el desarrollo de resistencia al BES. Además, se ha demostrado la degradación de BES en MFC. Por otro lado, a concentración 200 mM de BES la actividad metanógenica disminuye, pero resulta en un incremento de la recirculación de hidrógeno por homoacetogénesis debido a las condiciones favorables para estos microorganismos.

Por otro lado, se ha obtenido una alta intensidad de corriente (hasta 10 $A \cdot m^{-2}$) en una MEC inoculada con un cultivo puro de *Geoalkalibacter ferrihydriticus* a pH básico. Estos resultados sugieren la posibilidad de que las condiciones alcalinas puedan mejorar el rendimiento de las MXCs mediante la creación de un entorno más selectivo para los ARB. También se ha seleccionado, a partir de lodos anaerobios, una comunidad capaz de trabajar en condiciones de pH básico utilizando medio alcalino y un control del pH en línea. Se han obtenido unos rendimientos muy elevados tanto en MFC como en MEC (alrededor de 50 $mA \cdot m^{-2}$ y 2.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). El estudio de las poblaciones existentes muestra un elevado contenido en el género *Alkalibacter* en una MFC alcalina (37% de la comunidad bacteriana). La presencia de microorganismos del género *Geoalkalibacter* se ha confirmado a pH alto (9,3), particularmente en MEC (43%).

Finalmente, se ha desarrollado una estrategia con el propósito de obtener un cultivo sintrófico entre fermentadores y ARB capaz de usar el suero de leche como donador de electrones. Este es fermentado principalmente a acetato por bacterias del ácido láctico (el género *Enterococcus* comprendía el 22% de la comunidad bacteriana total) y otras bacterias fermentadoras de los géneros *Sphaerochaeta* y *Dysgonomonas*. La actividad exoelectrogénica se ha demostrado con la presencia de *Geobacter* sp. (37%), que consume el acetato como donador de electrones. Esta comunidad microbiana sintrófica ha sido capaz de degradar el suero de leche para producir directamente energía o H_2 (0.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). Por otro lado, las MXCs alimentadas con suero de leche han demostrado una capacidad intrínseca para inhibir la actividad metanogénica sin necesidad de utilizar otras estrategias de inhibición externas.

Resum

La necessitat d'energia renovable i la constant amenaça de l'escalfament global han motivat el desenvolupament de noves tecnologies més sostenibles que les actuals. Entre elles, els sistemes bioelectroquímics poden permetre un futur on l'energia i altres productes de valor afegit, com l'hidrogen, es puguin obtenir dels nostres residus orgànics. Així doncs, els sistemes bioelectroquímics encaixen perfectament en el nou paradigma actual pel que fa als residus, que han de ser valoritzats a més de ser tractats. Els sistemes bioelectroquímics, també coneguts amb el nom de cel·les electroquímiques microbianes (Microbial Electrochemical Cells, MXCs), combinen l'electroquímica amb el metabolisme d'un grup particular de microorganismes anomenats exoelectrògens o bacteris que respiren a partir d'un ànode (Anode Respiring Bacteria, ARB).

Entendre l'activitat metabòlica dels ARB i les seves condicions òptimes de creixement són de gran importància per tal de garantir el màxim rendiment de les MXCs. Així doncs, aquesta tesi estudia els sistemes bioelectroquímics i té com a objectiu desenvolupar, estudiar i millorar la comunitat microbiana de les MXCs per tal d'augmentar el seu rendiment.

Per a això, en aquesta tesi es van aplicar tècniques moleculars avançades per l'estudi de les poblacions microbianes com per exemple l'estudi metagenòmic del gen 16S ribosomal o la qPCR en temps real. Es van construir corbes qPCR per tal d'estudiar l'evolució dels ARB i de les diferents comunitats microbianes metanògenes existents en els sistemes d'estudi. A més a més, es van dur a terme experiments amb piroseqüenciació 454 per tal d'aprofundir en la composició de les comunitats microbianes que es troben als elèctrodes.

Es van dissenyar diferents muntatges experimentals per a estudiar les condicions d'operació que afavoreixen als ARB front altres microorganismes que disminueixen l'eficiència de les MXCs, com els metanògens o els bacteris homoacetogèns. El primer pas va ser l'enriquiment d'ARB sota diferents resistències externes en cel·les de combustible microbianes (Microbial Fuel Cells, MFC). Aquest estudi va demostrar que l'ànode inoculat en una MFC sota una resistència externa baixa (12 Ω) tenia un rendiment millor quan se'l traslladava a una cel·la d'electròlisi microbiana (Microbial Electrolysis Cell, MEC); és a dir, va donar major intensitat i major taxa de producció d'H_2 que altres MFC inoculades amb resistències externes superiors (220 i 1000 Ω). D'altra banda, les anàlisis de qPCR van confirmar que l'ús d'una resistència externa baixa en MFC comporta la formació d'una biopel·lícula anòdica amb major contingut de *Geobacter* (l'ARB més habitual).

Es va dur a terme un estudi en dues etapes per a conèixer les limitacions de l'ús a llarg termini de l'inhibidor més comú de la metanogènesi: el 2-brometàsulfonat (BES). En MFCs alimentades amb acetat, es va veure com el BES esdevenia ineficaç a llarg termini ja que no va poder evitar el creixement d'*Archaea* (concretament del gènere que usen hidrogen com a donador d'electrons,

Methanobrevibacter, de l'ordre *Methanobacteriales*). Fins i tot a nivells molt elevats de BES (fins a 200 mM) es va veure producció de metà, el que sembla indicar el desenvolupament d'una resistència al BES. A més a més, es va demostrar la degradació de BES en MFC. D'altra banda, a concentració 200 mM de BES, l'activitat metanogènica va disminuir però va resultar en un increment de la recirculació d'hidrogen per homoacetogènesi a causa de les condicions favorables per a aquests microorganismes.

D'altra banda, es va aconseguir una alta intensitat de corrent (fins a 10 $A \cdot m^{-2}$) en una MEC inoculada amb un cultiu pur de *Geoalkalibacter ferrihydriticus* a pH bàsic. Aquests resultats suggereixen la possibilitat de que les condicions alcalines puguin millorar el rendiment de les MXCs mitjançant la creació d'un entorn més selectiu pels ARB. També es va seleccionar, a partir de fangs anaerobis, una comunitat capaç de treballar en condicions de pH bàsic utilitzant medi alcalí i un control del pH en línia. Es van obtenir uns rendiments molt elevats tant en MFC com en MEC (al voltant de 50 $mA \cdot m^{-2}$ i 2.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). L'estudi de les poblacions existents va mostra un elevat contingut en el gènere *Alkalibacter* en una MFC alcalina (37% de la comunitat bacteriana). La presència de microorganismes del gènere *Geoalkalibacter* es va confirmar a pH alt (9,3), particularment en MEC (43%).

Finalment, es va desenvolupar una estratègia amb el propòsit d'obtenir un cultiu sintròfic entre fermentadors i ARB capaç d'usar el sèrum de llet com a donador d'electrons. Aquest era fermentat principalment a acetat per bacteris de l'àcid làctic (el gènere *Enterococcus* comprenia el 22% de la comunitat bacteriana total) i altres bacteris fermentadors dels gèneres *Sphaerochaeta* i *Dysgonomonas*. L'activitat exoelectrogènica es va demostrar amb la presència de *Geobacter* sp. (37%), que consumia l'acetat com a donador d'electrons. Aquesta comunitat microbiana sintròfica va ser capaç de degradar el sèrum de llet per a produir directament energia o H_2 (0.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). D'altra banda, les MXCs alimentades amb sèrum de llet van demostrar una capacitat intrínseca per inhibir l'activitat metanogènica sense necessitat d'utilitzar altres estratègies d'inhibició externes.

Riassunto

Il bisogno pressante di energie rinnovabili e la costante minaccia del riscaldamento globale hanno portato allo sviluppo di nuove tecnologie più sostenibili rispetto alle attuali. Tra queste, i sistemi bioelectrochimici possono permettere un futuro in cui l'energia e altri prodotti a valore aggiunto come l'idrogeno, possano essere ottenuti dai rifiuti organici. Quindi, i sistemi bioelectrochimici si adattano perfettamente nel paradigma corrente in cui i rifiuti devono essere valorizzati oltre ad essere trattati. I sistemi bioelectrochimici, noti anche con il nome di sistemi elettrochimici microbiani (Microbial Electrochemical Cells, MXCs), si combinano con il metabolismo di un particolare gruppo di microrganismi chiamati esoelettrogeni o ARB (acronimo inglese di Anode Respiring Bacteria).

Comprendere l'attività metabolica degli ARB e le condizioni di crescita ottimali di questi, sono di grande importanza per garantire la massima prestazione dei MXCs. Per cui, questa tesi studia i sistemi bioelectrochimici e mira a sviluppare, studiare e migliorare la comunità microbica dei MXCs con l'obiettivo di aumentarne le prestazioni energetiche. In questa tesi sono state applicate tecniche avanzate di biologia molecolare per l'indagine delle popolazioni microbiche come lo studio metagenomico del gene 16S ribosomiale o la PCR in tempo reale (qPCR). Vari esperimenti sono stati condotti con il pirosequenziamento 454 per approfondire la composizione delle comunità microbiche che crescono sulla superficie degli elettrodi.Inoltre, tre curve di qPCR sono state costruite per studiare l'evoluzione degli ARB e le diverse comunità microbiche metanogene presenti nei sistemi oggetto di ricerca.

Durante lo studio di ricerca sono stati progettati diversi disegni sperimentali per studiare le condizioni di operazione che favoriscono gli ARB rispetto ad altri microrganismi che sono noti per diminuire l'efficienza della MXCs: i metanogeni e i batteri omoacetogeni.

Il primo passo è stato l'arricchimento di ARB mediante l'uso di diverse resistenze esterne in celle a combustibile microbiche (Microbial Fuel Cells, MFC). Questo studio ha dimostrato che l'anodo in una MFC inoculata a bassa resistenza esterna (12 Ω) ha ottenuto prestazioni energetiche migliori quando è stato spostato in una cella di eletrolisi microbica (Microbial Electrolysis Cell, MEC); cioè ha dato maggiore intensità e velocità di produzione di H_2 superiore rispetto agli anodi inoculati in MFCs con resistenze esterne più alte (220 e 1000 Ω). Inoltre, l'analisi qPCR ha confermato che l'uso di una bassa resistenza esterna comporta la formazione di un biofilm anodico con maggior contenuto di *Geobacter* (ARB più comune).

Uno studio è stato condotto in due fasi per esplorare i limiti dell'utilizzo a lungo termine del inibitore più comune della metanogenesi: il 2-brometansulfonato (BES). L'inefficacia dell'utilizzo del BES durante lungo tempo è stata dimostrata in una MEC. In questo studio l'inibitore si è dimostrato incapace di limitare la crescita di *Archaea* e in particolare del genere *Methanobrevibacter*, dell'ordine *Methanobacteriales*, che utilizza l'idrogeno come donatore di elettroni. Anche a livelli molto elevati di BES (fino a 200 mM) è stata riportata una produzione di metano che suggerisce lo sviluppo di resistenza al BES. Inoltre, la degradazione del BES è stata dimostrata in MFC. D'altro canto, l'attività metanogenica si è ridotta quando la MEC è

stata sottoposta ad una concentrazione di BES di 200 mM, generando però il fenomeno del ricircolo dell'idrogeno causato dall'aumento dei batteri omoacetogeni che sono favoriti da queste condizioni.
Una corrente elevata è stato ottenuta (fino a 10 $A \cdot m^{-2}$) in una MEC inoculata con una colonia pura di *Geoalkalibacter ferrihydriticus* a pH basico. Questi risultati suggeriscono la possibilità di ottenere migliori prestazioni energetiche in un ambiente più selettivo come quello alcalino che può favorire gli ARB. Con questo scopo, è stata selezionata da fanghi anaerobici e mediante l'uso di condizioni alcaline e un controllo di pH on-line, una comunità microbiana capace di lavorare in condizioni di pH basico. Prestazioni energetiche molto elevate sono state ottenute sia in MFC che in MEC (circa 50 mA m^{-2} e 2.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). Lo studio delle popolazioni presenti mostra un elevato contenuto del genere *Alkalibacter* in una MFC alcalina (37% delle sequenze della popolazione batterica). L'elevata presenza di microrganismi del genus *Geoalkalibacter* (43%) è stato riscontrata in una MEC a pH elevato (9.3).
Infine, una strategia è stata sviluppata con lo scopo di ottenere una comunità microbica sintrofica di fermentatori ed ARB in grado di utilizzare il siero del latte, ottenuto dalla preparazione de formaggio, come un donatore di elettroni. Questo è stato principalmente fermentato dai batteri lattici (il genere *Enterococcus* costituiva il 22% delle sequenze della comunità batterica totale) e altri fermentatori dei generi *Dysgonomonas* e *Sphaerochaeta*. L'attività esoelettrogena è stata dimostrata dalla presenza di *Geobacter* sp. (37%) che consuma acetato come un donatore di elettroni. Questa comunità microbica sintrofica è stata in grado di degradare il siero di latte per produrre direttamente energia o H_2 (0.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$). Inoltre, in queste MXCs è stata confermata la capacità intrinseca del siero di inibire l'attività metanogenica senza utilizzare altre strategie esterne di inibizione.

Table of Contents

CHAPTER 1 General Introduction 2
1.1. European energy situation 3
1.2. Hydrogen as energy vector 4
1.3. Bioelectrochemical systems 4
Focus 1. Voltage losses 7
1.4. Anode Respiring Bacteria (ARB) 9
Focus 2. ARB Electron Transport 10
1.5. Double or single chamber MEC? 11
Focus 3. Methanogenesis and its inhibition in MXCs 13
1.6. Metagenomics 14
1.7. 16S rRNA gene 15
1.8. Polymerase chain reaction (PCR) 17
1.9. Quantitative real-time polymerase chain reaction (qPCR) 17
1.10. High-throughput 16S rRNA gene pyrosequencing 18
1.11. Research motivations and thesis overview 21
1.12. Background of the research group in bioelectrochemical systems 21
1.13. Objectives 22
1.14. Thesis overview 23
CHAPTER 2 Materials and Methods 25
2.1. Inoculum-MFC 27
2.2. Cube MFC and inoculation protocol 28
2.3. Cube MEC 28
2.4. Medium 29
2.5. Electrochemical calculations 30
2.6. Gas chromatography 31
CHAPTER 3 DNA-based analysis of the microbial community structure in single-chamber MXCs 33
Motivation 35
3.1. Quantitative real-time PCR (qPCR) 36
3.1.1. qPCR standard curves generation procedure 36

3.1.2. Obtaining qPCR standard curves 41
3.2. Use of qPCR to evaluate the effect of different external resistances 44
3.2.1. Specific experimental procedures for section 3.2 44
3.2.2. Results and discussion 46
3.3.Conclusions 51
CHAPTER 4 Limitations of the long-term utilization of 2-bromoethanesulfonate as methanogenic inhibitor in bioelectrochemical systems 53
Motivation 55
4.1. Microbial community analysis in a long-term membrane-less microbial electrolysis cell with hydrogen and methane production 56
4.1.1. Specific experimental procedures for section 4.1 56
4.1.2. Results 60
4.1.3. Discussion 68
4.2. BES degradation in bioelectrochemical systems 71
4.2.1. Specific experimental procedures for section 4.2 71
4.2.2. Results and discussion 73
4.3.Conclusions 78
CHAPTER 5 Pure and mixed alkaline cultures MXCs: development and study 79
Motivation 81
5.1. Opportunities of *Geoalkalibacter Ferrihydriticus* for bioelectrochemical hydrogen production 82
5.1.1. Specific experimental procedures for section 5.1 82
5.1.2. Results and discussion 84
5.2. Bioelectrochemical electrical current and hydrogen production under alkaline conditions .. 91
5.2.1. Specific experimental procedures for section 5.2 91
5.2.2. Results and discussion 94
5.3.Conclusions 100
CHAPTER 6 Cheese whey in MECs: a suitable substrate for hydrogen production without methane 103

Motivation 105
6.1. Specific experimental procedures 106
6.2. Results and discussion 108
6.3. Conclusions 116
CHAPTER 7 General Conclusions 117
Future Work 123
Glossary 127
List of Acronyms & Abbreviations 133
References 139
Curriculum Vitae 163

CHAPTER 1

General Introduction

1.1.European energy situation

In 2006, the Council of the European Union during the Brussels European Council [1] analyzed the difficult energetic situation of Europe in the last years. The rising of global energy demand and the need to reduce the growing threats of climate change, suggested the need of finding alternative energy to supply the scarcity of fossil fuels, to decrease CO_2 and greenhouse gases emissions and to abate the European dependency from importing gas (24.5 % in 2011 [2]) and oil (62.7 % of crude oil and derived products in 2011 [2]). On these bases, European Union (EU) aims for 20% of all energy to come from renewable energy sources by 2020 [1].

The Eurostat analysis of 2012 [3] determined that, during the last years, the share of energy from renewable sources, which was 8.1 % of total energy in 2004 and 12.5 % in 2010, continued to grow despite the financial and economical crisis. However, the next Eurostat study of 2014 [2] showed that energy from renewable sources and waste (which includes non-renewable industrial and municipal waste) in EU was only 11.7%. In this study, it is also possible to observe that electricity generation from renewable including hydro-electricity and waste supplied 20.5 % (16.1% was hydro-electricity) of the world's electricity in 2011, with a slightly higher share recorded in the EU (22.0 %).

Although hydro-electricity dominates electricity generation from renewable and waste, it is not the most environmentally friendly source due to its high costs to build power plants and its environmental consequences related to interventions in nature (damming of water, changed water flow and the construction of roads and power lines) [4]. Indeed, renewable energies (such wind, sun and biodiesel) and biowastes are preferred alternative sources. Biowastes have the advantages, unlike biodiesel, of not competing with the food chain and that the valorization of the residues is accomplished. Hence, the exploitation of wastes for conversion to fuels, power, heat and added-value chemicals is the new focus of energy research. Anaerobic digestion produces gases (mainly methane) that can be used as a fuel. It is, currently, the only technology that has proven capable of extracting energy from wastewaters on a commercial scale [5].

In this frame, energy and H_2 production from industrial and municipal wastewater using bioelectrochemical systems is arousing scientific interest and it could be really convenient to supply, for example, the high energetic and economic costs of wastewater treatment [6].

1.2.Hydrogen as energy vector

Hydrogen (H_2) is a preferred alternative energy source since it is a clean and renewable energy carrier, without an impact on the greenhouse gas emission during its energy generation step and a high combustion heat (120 kJ/g) when compared to other possible biofuels CH_4, (50 kJ/g) or ethanol, (26.8 kJ/g) [7]. In addition, H_2 can be very efficiently converted into electricity through fuel cells.

Nowadays, most H_2 is produced via steam reforming, a non-sustainable option. Biological hydrogen production, which offers the possibility of being renewable and carbon neutral, can be achieved by photosynthesis, fermentation, and microbial electrolysis cells [8]. Among all the current biological H_2 production techniques, the utilization of bioelectrochemical systems is very attractive because high conversions can be achieved. Dark fermentation would only produce a maximum amount of 4 moles of H_2 per mole of glucose while most of the electronic content in substrate, except for the growth requirements, could be recovered using bioelectrochemical systems [9].

1.3.Bioelectrochemical systems

Bioelectrochemistry is an emerging field of research that combines the metabolism of microorganisms with electrochemistry. There are two principal types of bioelectrochemical system that are also called microbial electrochemical cells (MXCs):

1) Microbial fuel cell (MFC), aiming at electricity production (Figure 1.1);

2) Microbial electrolysis cell (MEC), aiming at biohydrogen production (Figure 1.2).

An MFC consists of an anode, a cathode and an electrical circuit. Oxidation occurs in the anode where anode respiring bacteria (ARB, see section 1.4 for further details) convert organic matter into CO_2, protons and electrons and transfer the electrons to an insoluble conductive anode. Protons are released into the medium and electrons flow through an electrical circuit to the cathode. A favorable reduction process occurs in the cathode (generally oxygen reduction), which makes the process thermodynamically feasible and, thus, electricity is generated.

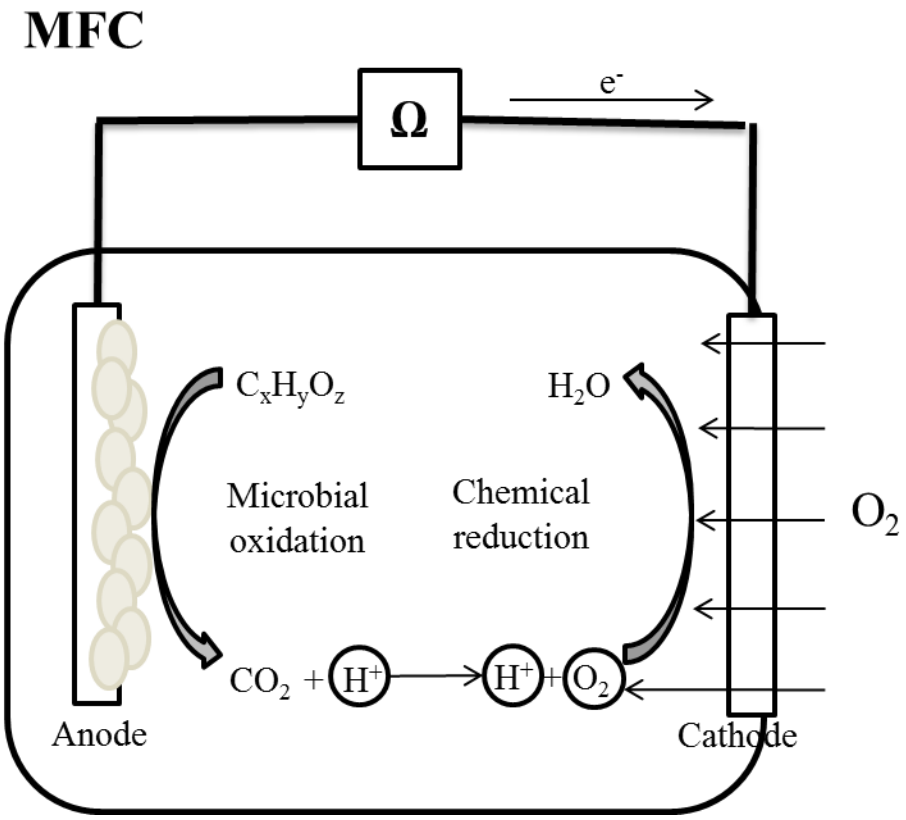

Figure 1.1. Schematic representation of a single chamber air cathode microbial fuel cell (MFC)

When acetate is the electron donor the reactions occurring in each electrode can be expressed as:

Anode, oxidation reaction: $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 8e^- + 9H^+$

($E_{HCO3-|CH3COO-}$ = -0.28 V vs. SHE)

Cathode, reduction reaction: $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$

($E_{O2|H2O}$ = 0.82 V vs. SHE)

With an overall cell electromotive force of $E_{emf} = E_{cathode} - E_{anode}$ = 1.1 V

In an MEC, the cathodic reaction is hydrogen formation from the protons and the electrons transferred. Thermodynamics indicate that the process is not spontaneous and, thus, that an applied potential is required to drive hydrogen production [6,10].

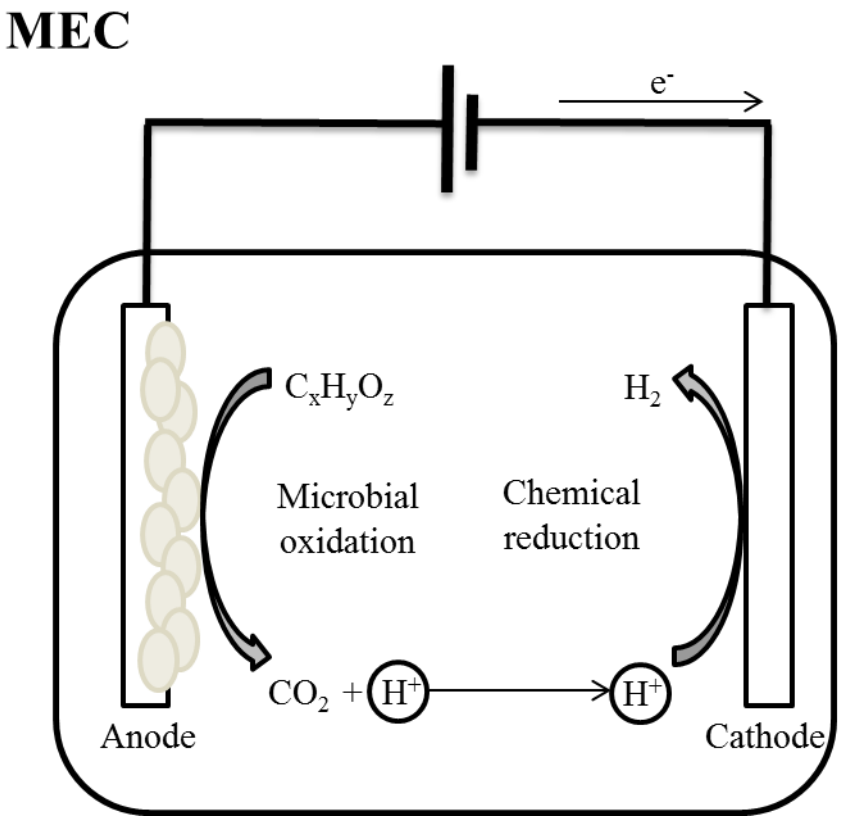

Figure 1.2. Schematic representation of a single chamber microbial electrolysis cell (MEC)

Cathode, reduction reaction: $2H^+ + 2e^- \rightarrow H_2$

($E_{H+/H2}$ = -0.41 V vs. SHE)

With an overall cell electromotive force of $E_{emf} = E_{cathode} - E_{anode}$ = -0.13 V.

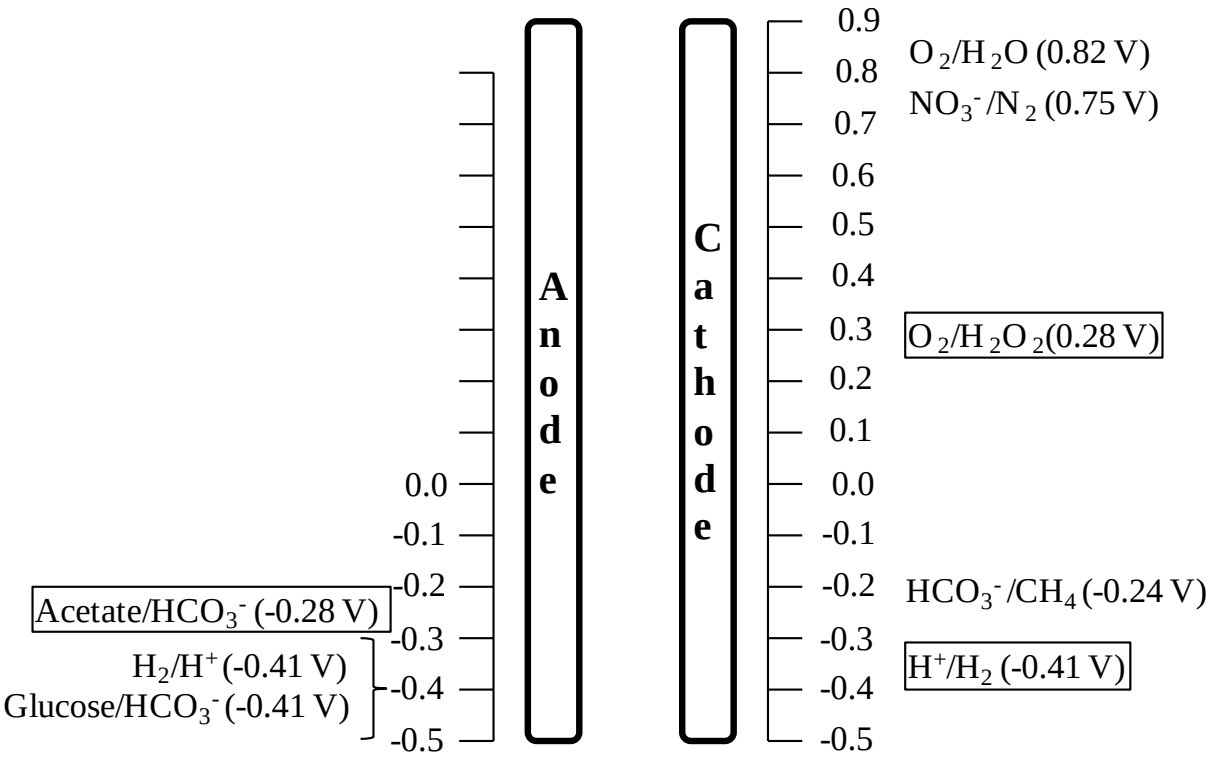

Figure 1.3. Scale of theoretical potentials associated to redox processes at pH=7 and T=298 K (versus standard hydrogen electrode) [11].

Although about 1.1 V can be theoretically produced from acetate in an MFC [12], in practice, the voltage that can be obtained is lower due to the high existing voltage losses. In MEC only 0.13 V (at 298 K and pH 7) should theoretically be enough for H_2 production from acetate (Figure 1.3) [13], but in practice the voltage that is required is higher (0.5 to 1 V is needed under lab conditions) also due to the voltage losses [14].

Focus 1. Voltage losses

As already explained, the voltage that can be theoretically achieved in an MFC or that it is required in an MEC differs from the thermodynamic values due to a whole variety of voltage losses occurring in the system (known as overpotentials). These voltage losses (Figure 1.4) can

be divided into:

- ***activation losses*** that represent the voltage losses required to initiate a reaction. They depend on the type of catalyst used: a better catalyst decreases the activation energy and hence causes lower activation losses.

- ***ohmic losses*** that are the resistance to the flow of electrons in electrodes and connections, the resistance to the flow of ions in the electrolytes and the membrane (if present). due to the *internal resistance* of the bioelectrochemical system itself: The reduction of the space between the electrodes, increasing of solution conductivity and use of membrane-less MXCs can help to reduce ohmic losses

- ***bacterial metabolic losses*** due to the energy gain for bacteria by transporting electrons from a substrate to a final electron acceptor.

- ***concentration or mass transfer losses*** related to mass transport limitations of substrates and products.

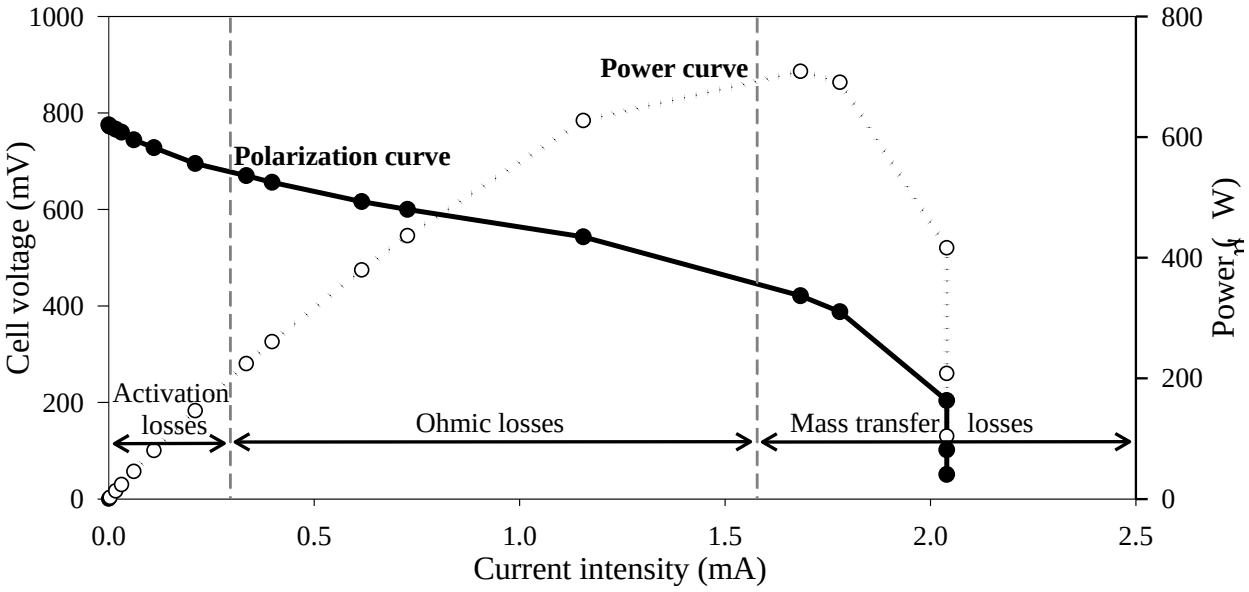

Figure 1.4. The different types of voltage losses are shown on polarization and power curves obtained with a multi-resistance board which allowed changing the external resistance of the cell (17 different resistors between 470 kΩ to 25 Ω). Current intensity was calculated by Ohm's Law and power by the following relationship: $P = V \cdot I$ where P (W) is the power output.

1.4.Anode Respiring Bacteria (ARB)

MXCs rely on the presence of the particular biochemical pathway of a group of bacteria named anode respiring bacteria(ARB) which are able to transfer electrons outside the cell. ARB are also called exoelectrogens, “exo-” for exocellular and “electrogens” based on the ability to directly transfer electrons to a chemical or material that is not the immediate electron acceptor [6]. ARB are able to oxidize organic matter under anaerobic conditions and to transfer the electrons gained in their metabolism out of the cell using a solid electrode (the anode) as electron acceptor [15].

The first step in ARB metabolism (Figure 1.5) consists of the oxidation of electron donor (i.e. acetate or other organic matter) and the generation of electrons which are transferred into the bacterial cell, by using an electron carrier such as the coenzyme NADH. The coenzyme is oxidized to generate energy for the cells and the electrons are transferred to the membrane transport proteins that are part of the electron transport chain. At this point, electrons are transferred to an external electron acceptor [16], which will depend on the specific electron transport mechanism (Figure 1.6).

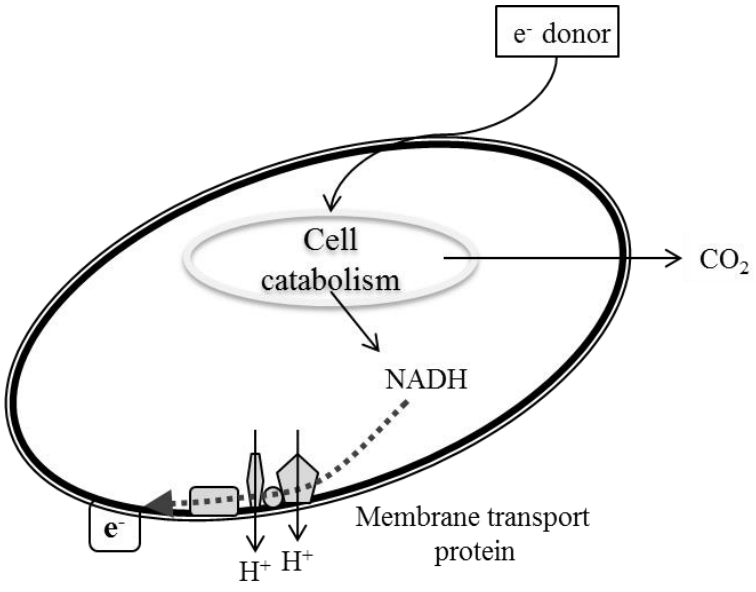

Figure 1.5. Schematic representation of ARB catabolism and e^- production [16].

Examples of microorganisms with exoelectrogenic capabilities are: *Geobacter metallireducens* [17], *Desulfuromonas acetoxidans* [17], *Geobacter sulfurreducens* [18], *Rhodoferax ferrireducens* [19], *Shewanella oneidensis* [20], *Pseudomonas aeruginosa* [21], *Geothrix fermentans* [22], *Thermincola ferriacetica* [23], *Geoalkalibacter ferrihydriticus and subterraneus* [24]. The most studied ARB genera are *Geobacter* and *Shewanella* [6,25,26]. The genera of *Geobacteraceae* family, which includes *Geobacter* [27,28], are often found in the anodic biofilm of acetate-fed MXCs. The anaerobic condition and their ability to colonize the electrode surface give them a competitive advantage over other microorganism in MXCs [18]. Several different ARB can be found in MXCs depending on the inoculums, the substrate and the experimental conditions [25,26,29].

Focus 2. ARB Electron Transport

ARB are so far known to transfer electrons to a surface via three mechanisms (Figure 1.6) [16]:

- direct interaction with solid electron acceptor;

- matrix generation to connect the cell wall and the electrode (nanowires);

- electron shuttling via chemical mediators.

The first mechanism is supported by the presence of extracellular cytochromes that can interact directly with the solid surface to carry out respiration.

The second mechanism proposes the presence of a conductive extracellular biofilm matrix that connects the bacteria to the solid surface. This mechanism is supported by the presence of conductive nanowires (similar to pili bacteria) which have the capability to conduct electrons.

The third mechanism proposes the presence of a chemical compound (called shuttle) that, after being reduced in the ARB cells, it carries electrons from the bacteria by diffusive transport to the electrode surface to finally discharge its electrons. Some studies demonstrated that the different transfer electrons mechanisms are used depending on the ARB species and the environmental conditions [30]. *Shewanella oneidensis* is capable of producing shuttles such as flavin

mononucleotide and riboflavin [31,32] and also bacterial nanowires, which are electrically conductive pilus-like appendages in direct response to electron-acceptor limitation [33]. Other organisms, such as *Geothrix ferementans* [34] and *Pseudomonas* species [35] also produce electron shuttles. Moreover, it was confirmed that *Geobacter sulfurreducens* biofilm exhibited more than one pathway of electron transfer to an anode, depending on the poised anode potential [36]. Recently, species of *Geolkalibacter* genus have been demonstrated to perform long-range electron transfer through electrode-attached biofilms and not through soluble electron shuttles [24].

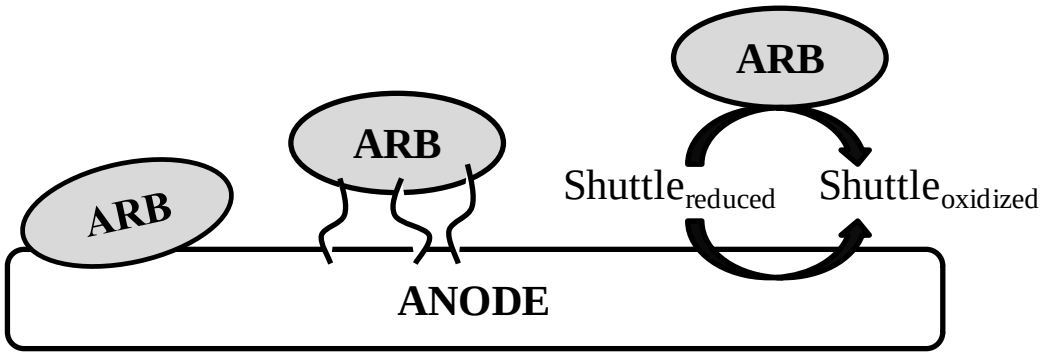

Figure 1.6. Schematic of three electrons transport mechanisms used by ARB: direct electron transfer, nanowires and electron shuttle [16].

1.5.Double or single chamber MEC?

The production of hydrogen in MEC has given very promising results at lab-scale but its success is undermined by two main problems: the presence of hydrogen scavengers and the need of high applied voltages [37].

In a double-chamber MXC, both the anodic and cathodic processes can be physically separated by an ion exchange membrane, selectively allowing cations flow (cation exchange membrane, CEM) or anions flow (anion exchange membrane, AEM). The use of membranes in double-chamber MEC configuration is useful to prevent methanogenesis, H_2-recycling [38] and to avoid

impurities in H_2 [10], due to the separation between the anodic chamber where is the substrate (i.e. acetate) and the cathodic chamber where the product (H_2) is present.

On the other hand, it increases the cost and the maintenance due to the need of a higher applied potential to compensate the increase of potential losses derived by the membrane presence. The voltage losses associated with an ionic exchange membrane were estimated between 0.26 and 0.38 V [10].

A single-chamber MEC configuration, also called membrane-less MEC, has been proposed [39] to obtain a simpler configuration and maintenance. On the other hand, the success of membrane-less MECs is challenged by the bacteria colonizing each electrode (Figure 1.7). MEC conditions (i.e. anaerobic environments with an organic matter as electron donor, high CO_2 and H_2 concentration) may enable the proliferation of other microorganism, such as methanogens or homoacetogens [9].

Methanogens are greatly favored by the excess of biodegradable carbon and anaerobic conditions. Methanogens that can produce CH_4 from CO_2 and H_2 are called hydrogenotrophic and methanogens that can synthesize CH_4 using acetate are called acetoclastic [40,41].

Homoacetogenic autotrophic bacteria are strictly anaerobic microorganisms that catalyze the formation of acetate from different substrates [42]. They can synthesize acetate by recycling the H_2 and CO_2 generated by ARB and generate a failure in H_2-producing MEC [38].

The complexity of the microbiological communities present in MXCs claims to be further investigated. Thus, metagenomic technologies were recently used in different studies [43–49].

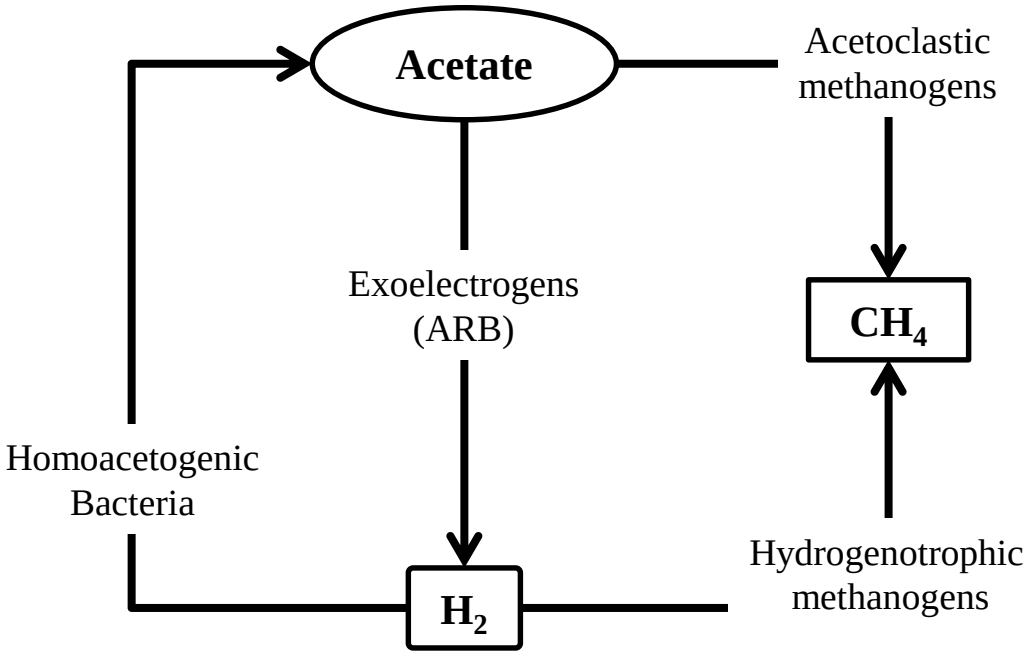

Figure 1.7. H_2-production and H_2 scavengers in MEC systems

Focus 3. Methanogenesis and its inhibition in MXCs

Methanogens are microorganisms included in *Archaea* domain that produce methane as a metabolic byproduct under anaerobic conditions. Methanogens presence is one of the most important reported failures in MXCs and especially in H_2-producing MEC [44,50,51]. Thus, understanding and inhibiting methanogenesis in these systems is essential in view of its future implementation. Aetoclastic methanogens compete with exoelectrogens for the same electron donor (acetate), whereas hydrogenotrophic methanogens use the H_2 produced in the reactor. Hydrogenotrophic methanogens are particularly relevant in membrane-less MECs since the hydrogen produced in the cathode is a preferred electron donor for methane production [38]. Another biochemical pathway was recently suggested and it includes methanogens that could directly use electrons from the cathode to produce CH_4, i.e. electromethanogenesis [52,53].

Therefore, methanogenesis suppression in bioelectrochemical systems is essential for their optimal performance and this issue has therefore been the focus of much recent research [54,55]. Previous studies have attempted to inhibit methanogenesis in bioelectrochemical systems using

pH and/or temperature shocks and periodic expositions to oxygen. However, these approaches were less successful than chemical inhibitors, such as 2-bromoethanesulfonate (BES) [54]. BES is a structural analog of Coenzyme M (CoM) (Figure 1.8) and inhibits effectively the methyl-CoM reductase reaction [56–58]. Because of its high effectiveness regardless of the electron donor, BES is the most commonly applied chemical inhibitor in laboratory-scale bioelectrochemical systems. BES ($Br\text{-}CH_2CH_2\text{-}SO_3Na$) is a sulfonate salt composed of a sulfite group (SO_3^{2-}) and an ethyl group ($C_2H_5^-$), with one H substituted by a bromine (Br) atom. There is yet no clear consensus about the most appropriate BES concentration for inhibition of methanogenesis, with a wide range of concentrations (0.25 to 50 mM) being reported for anaerobic suspended biomass and bioelectrochemical systems [43,51].

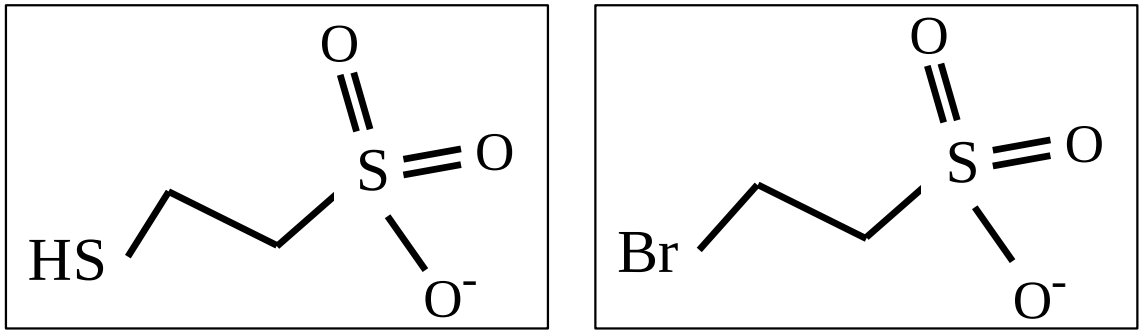

Figure 1.8. Coenzyme M (left) and 2-bromoethanesulfonate (right) chemical structures.

1.6.Metagenomics

The term “metagenomics” literally means “beyond the genome” [59] and it was first published in a study of soil microbes in 1998 [60]. Then, the definition varied to embrace any study which includes a whole community analysis [59]. Metagenomics allows the study of the genomes of the microorganism of a microbial community directly by extracting the DNA from environmental samples and without culturing in the laboratory [61]. This culture-independent technique offers the opportunity to study the total genetic pool, including the microorganism that cannot be cultured, permitting the analysis of the structure of microbial communities and also revealing how individual groups contribute to the biological activity of the community [62].

Gene-targeted metagenomics (also called metagene analysis) is a novel approach that permits to simplify metagenomics study using a particular gene of particular interest as target [63]. 16S ribosomal ribonucleic acid (rRNA) gene is widely used as a phylogenetic marker gene for prokaryotic metagenomics studies [64].

1.7.16S rRNA gene

Functional ribosomes are composed by three different classes of RNA molecules and 50 proteins [65]. In prokaryotic cells, the rRNA genes are transcribed from the ribosomal operon as 30S rRNA precursor molecules and then cut into 16S, 23S, and 5S rRNA molecules (Figure 1.9) [66].

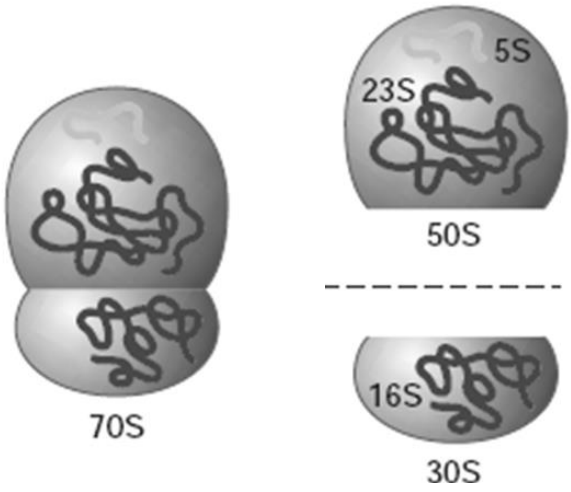

Figure 1.9. Schematic representation of prokaryotic ribosome.

The three rRNA genes are considered highly conserved [67], which means that these sequences changed only slightly or not at all by evolution, despite speciation. In 1987, Dr. Carl Woese proposed the use of 16S rRNA gene, which is the most conserved, to the reconstruction of the tree of life (Figure 1.10) [68].

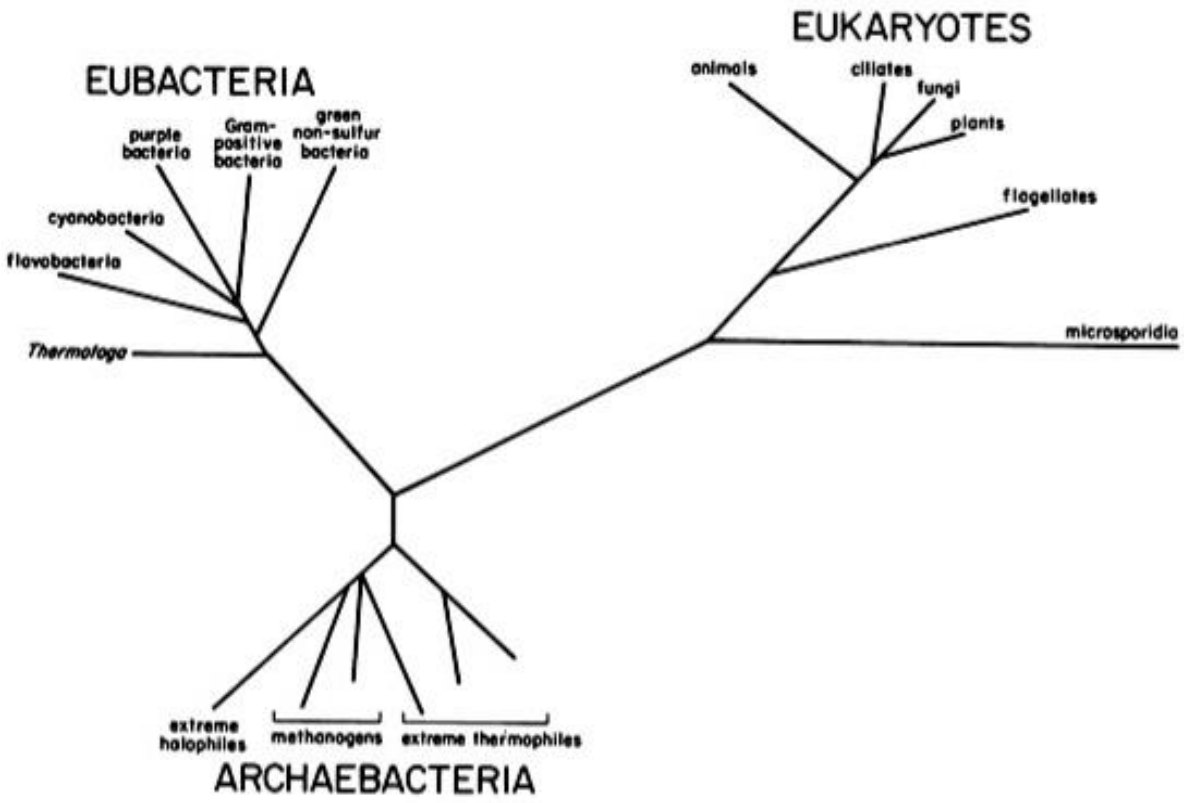

Figure 1.10. The initial tree of rRNAs shows three distinct founding domains [68]

16S rRNA gene contents nine variable regions that are considered group or species specific conserved (Figure 1.11) [69].

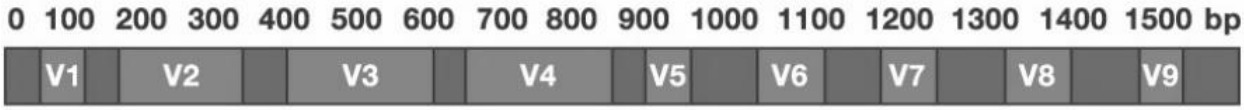

Figure 1.11. Graphical representation of variable (light grey) and conserved (dark grey) regions of 16S rRNA gene

When used to explore the diversity of prokaryotic communities, 16S rRNA gene hypervariable regions V1-V6 are commonly used [70]

16S rRNA gene-targeted metagenomic studies were included in this thesis.

1.8.Polymerase chain reaction (PCR)

PCR is a technique for amplifying DNA with the aim of creating, for example, multiple copies of a piece of DNA or to isolate DNA sequences. The first step is the denaturation by increasing the temperature to 95ºC. After the separation of the double-strand DNA target the temperature is decreased to ~60°C (it depends of the primers). This allows the primers to bind to the gene of interest (annealing). Then a thermostable polymerase binds the DNA and it synthesizes a complementary sequence of bases to the single-strand DNA. The temperature is raised to 72°C to allow the enzyme to work faster and to finalize the synthesis from forward to reverse primers (elongation). This temperature change is repeated through around 35-40 “cycles” by using a thermocycler. Thus one copy becomes 2, 2 become 4, 4 become 8, and so on until billions of copies are created [66,71].

1.9.Quantitative real-time polymerase chain reaction (qPCR)

qPCR is a recent technique that permits the direct quantification of the copy number contents in a DNA sample. qPCR is based on traditional PCR. The advantage of qPCR is that the use of a fluorophore permits to quantify the exact number of copies of DNA target, content in the initial sample [72]. There are two common strategies:

- SYBR Green is a dye that double-strand DNA. When SYBR Green dye binds to double-strand DNA, the intensity of the fluorescent emissions increases and it is reported by thermocycler that contains sensors for measuring the fluorescence. As more double-strand amplicons are produced, SYBR Green dye signal increases [73].

- TaqMan® probe (Figure 1.12) is a DNA sequence designed to bind selectively the target DNA among forward and reverse primers. A fluorescent reporter is bind in 5' position and a quencher of fluorescence is bind in 3' position of the TaqMan® probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence. The polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser [74].

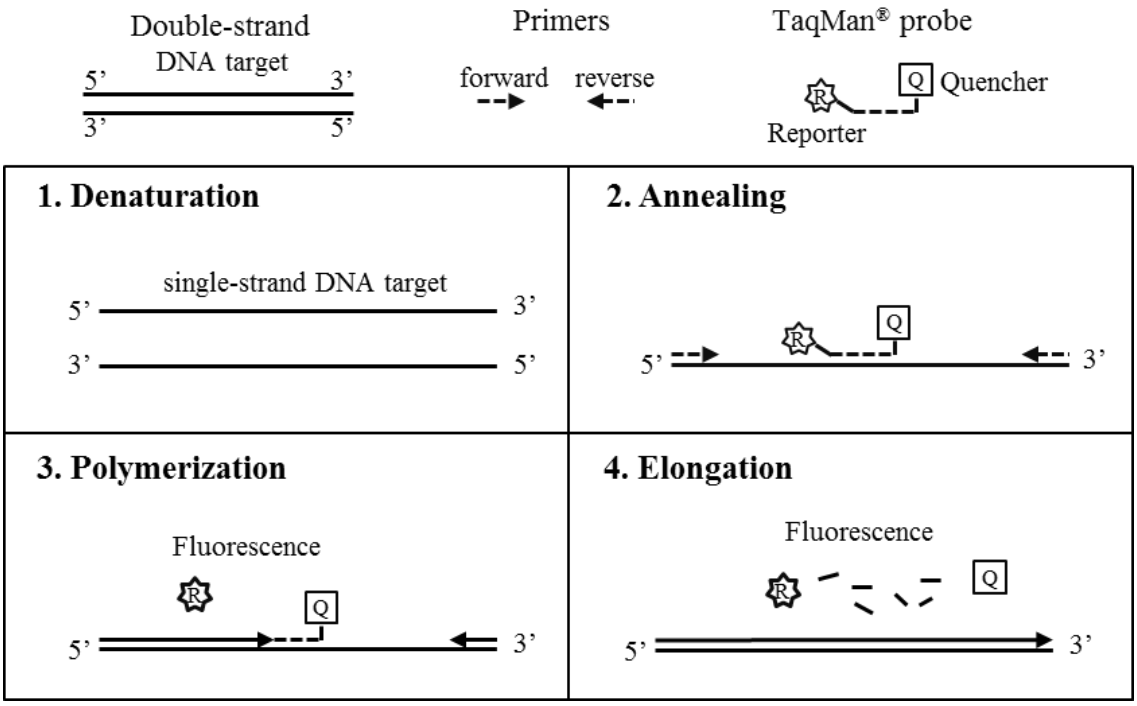

Figure 1.12. Schematic representation of the principal steps of TaqMan® probe quantitative real-time polymerase chain reaction.

1.10. High-throughput 16S rRNA gene pyrosequencing

Pyrosequencing technology permits to detect, in a single run, the whole set of microorganism contained in a microbial community, regardless of the abundance. Pyrosequencing of the 16S

rRNA gene has been proved as an optimum technology for metagenomics analysis of samples from diverse environments [69,70,75,76].

The 454 technology is probably the most used pyrosequencing platform. This technology involves a combination of emulsion PCR and pyrosequencing [70]. Amplicons of partial 16S rRNA gene sequences are attached and sequenced on microscopic beads placed in a PicoTiterPlate™ (PTP). The plate consists of approximately one million picoliter-sized wells [77], and in each well is placed one bead carrying a copy of a single-stranded DNA fragment to be sequenced [78]. Sulfurylase and luciferase molecules are also packed into the well and they permit light generation [79]. After the arrangement of the beads into wells the reaction starts and the nucleotide reagents (T, A, C, G) repeatedly flowing over the plate.

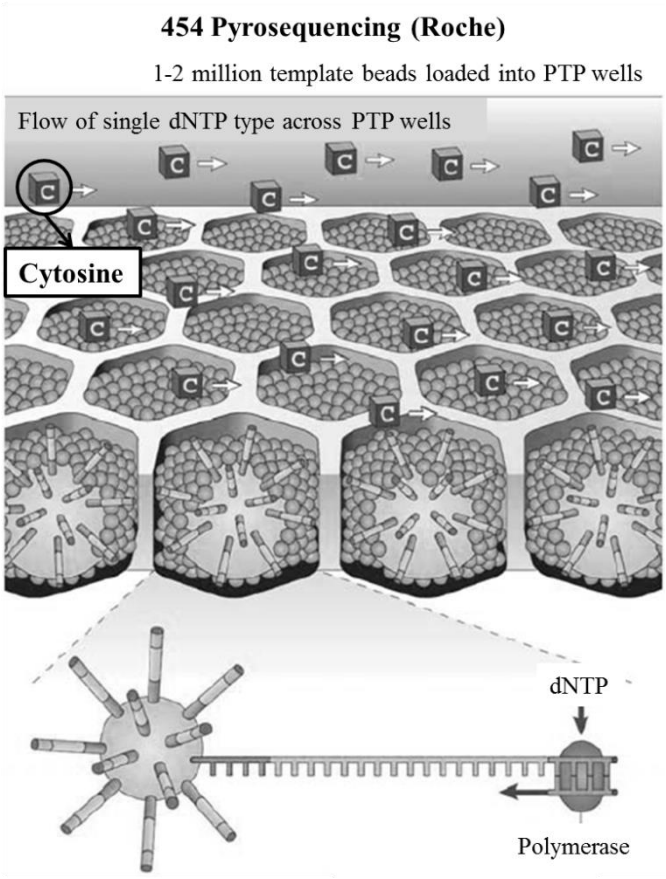

Figure 1.13. 454/Roche - pyrosequencing. After the arrangement of 1-2 milion beads into PTP wells the nucleotides (Cytosine in this case) flow over the plate and are incorporated in the growing chain of DNA [80].

Nucleotide incorporation in the growing chain of DNA is detected by the associated release of inorganic pyrophosphate and the generation of photons [81]. All the light signals are recorded by a camera and the light intensities are converted in a flowgram.

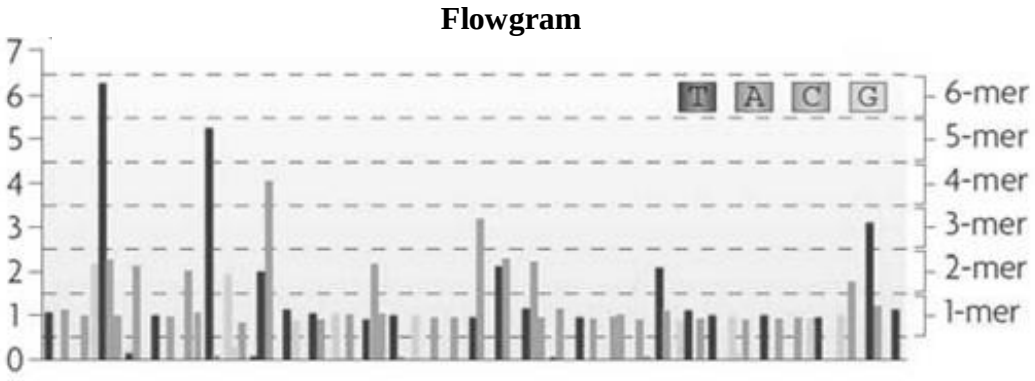

Figure 1.14. Example of a flowgram [80].

The results of pyrosequencing are filtered using bioinformatic tools and compared with databases. The Ribosomal Database Project (RDP) is a major repository of bacterial and archaeal 16S rRNA sequences. The RDP is also integrated with the Pyrosequencing Pipeline [82] which contains alignments and analysis tools for browsing, classification, probe checking, sequence matching, quality trimming, phylotype clustering, as well as statistical and ecological metrics like rarefaction curves, and diversity index and richness estimations among others [77,82,83].

According to previous studies, high-throughput 16S rRNA gene 454-pyrosequencing was used in this thesis for the characterization of microbial communities in MXCs [29,44,45] and the results were analyzed by using RDP Pyrosequencing Pipeline [82].

1.11. Research motivations and thesis overview

This thesis is framed in one of the research lines of the GENOCOV group (Research Group on Biological Treatment of Liquid and Gas Effluents; www.genocov.com) in the Department of Chemical Engineering at the Universitat Autònoma de Barcelona. GENOCOV was established in 1994 with the aim to conduct research on improving the biological wastewater treatment systems. In 2009, the research line on Bioelectrochemical Systems was born with the focus of recovering most of the chemical energy contained in wastewater to generate electricity and hydrogen.

This is the third thesis in the research line and was started in 2011 with the aim to study the microbiological communities developed in bioelectrochemical systems.

1.12. Background of the research group in bioelectrochemical systems

Although the research line of Bioelectrochemical Systems in GENOCOV was created only six years ago, some achievements have already been obtained. Moreover, most of the analytical techniques for volatile fatty acids and hydrogen/methane analysis were already set up. The group had also incorporated polarization curves as a routine technique for the analysis and characterization of MFC through a multi-resistance board and had studied many different cell configurations at lab scale and the use of potentiostatic techniques for both MFC and MEC.

A low-cost procedure to select ARB from anaerobic sludge was published [84]. Significant research was conducted in the group in the field such as the development and microbiological analysis of a syntrophic consortia between fermentative bacteria and ARB, able to degrade different complex carbon sources (methanol, milk and starch) [46,47]. A useful electron balances approach to study hydrogen scavengers in MEC was developed and validated [38]. Moreover, a strategy to avoid methanogenesis in MEC by reducing the hydrogen retention time was developed [85]. The performance of different cathode materials at neutral and basic pH was also assessed [86]. 0.58 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$ of hydrogen at an applied voltage of only 0.2 V were obtained by controlling the anodic pH 7.5 and the cathodic pH at 2.0 [87].

During this period, the group was funded by grants from the Spanish Government (Explora-Ingenio 2010, CTQ2009-06842-E/PPQ) and the Catalan Government (VALTEC13-1-0140) and by an agreement with Carburos Metálicos S.A (Air Products Group).

1.13. Objectives

This work investigates the different microbiological communities that grow in bioelectrochemical systems. Understanding the presence and the function of each single microbial community present can help to the development of single chamber microbial electrolysis cell for hydrogen production from synthetic and real wastewater. With this aim the following objectives were set:

- Optimization of specific protocols to improve the use of bio-molecular techniques aiming at the microbiological analysis.

- Development of strategies to enhance the growth and activity of exoelectrogenic bacteria on anodic biofilms.

- Metagenomic study and quantitative PCR of biofilms that grow in MXCs electrodes and media.- Long term effects of BES in the microbial communities.

- Understanding the fate of hydrogen in membrane-less MEC through the microbiological analysis.

- Minimization of methanogenic and homoacetogenic activity without addition of chemical inhibitors by generating extreme environmental conditions.

- Determination of the opportunities of using real substrates, as cheese whey, for electricity and hydrogen production in single chamber MXCs.

1.14. Thesis overview

This document is divided into six chapters.

- Chapter 1 comprises a general introduction to the topic, a literature review of the state of the art and the main objectives of this thesis.

- Chapter 2 (Materials and Methods) comprises the lab setups description and all common procedures used in this work.

The main results of this thesis are presented in Chapters 3 to 6.

- Chapter 3 includes the set-up of qPCR for the microbial analysis in single-chamber bioelectrochemical systems. The developed methodology is used to study MXCs inoculated at different external resistances.

- Chapter 4 discusses the use, limits and problems of the most common chemical inhibitor for methanogens: 2-bromoethanesulfonate.

- Chapter 5 describes the selection of alkaline microbial communities in MXCs.

- Chapter 6 details the use of cheese whey for electricity and hydrogen production.

Following, Chapter 7 presents the general conclusions. Additionally, references, glossary and list of abbreviations are included.

CHAPTER 2

Materials and Methods

The general materials and methods used in this thesis are presented in this chapter. The specific experimental procedures are explained in each chapter.

2.1.Inoculum-MFC

Inoculum-MFC (i-MFC; Figure 2.1) was an air-cathode MFC used to enrich the community in ARB. The wasted medium from i-MFC was used to inoculate the cube-MFCs.

i-MFC consisted in a 400 ml glass vessel with a lateral aperture (6.3 cm of diameter) for the cathode assembling. The cathode was a graphite fiber cloth (7.5 cm of diameter) treated as described in Cheng et al. [88]. It had a PTFE diffusion layer which permited oxygen diffusion into the cell while prevented liquid leakages, and a catalytic layer coated with platinum (5 mg Pt/cm^2, ElectroChem Inc.).

The anode (area 0.8 m^2) was a graphite handmade brush made as described in Ribot-Llobet et al. [84] with graphite fibers twisted with a titanium wire. The anode was thermally treated at 450 $^{\circ}$C for 30 minutes to produce micro fractures in the fiber to increase microbial adhesion [89]. Titanium wire was pierced on a cap provided with a silicone septum and it was used to connect the anode and the cathode by using an external resistance. The intensity produced was monitored online by quantifying the voltage drop across an external resistance serially connected to the circuit.

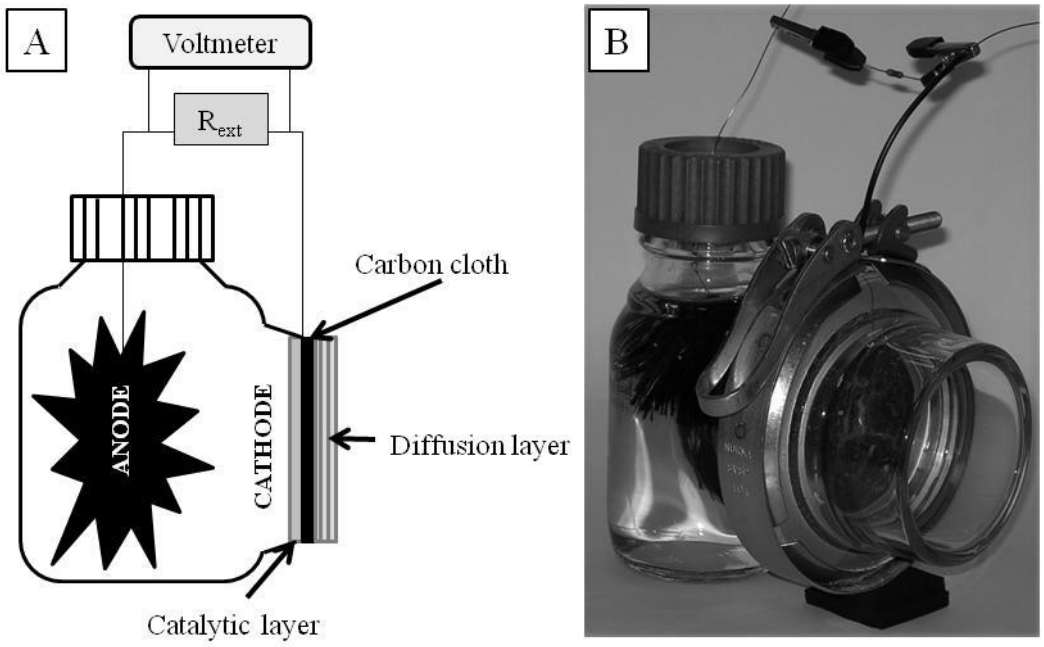

Figure 2.1. Schematic representation (A) and real image (B) of *inoculum*-MFC

2.2.Cube MFC and inoculation protocol

Cube-MFC (c-MFC; figure 2.2) was designed and built as described in Montpart et al. [47] by using 28 mL methacrylate cube vessels, with an air-cathode and an anode spaced 2.5 cm apart connected through an external resistance. One of the end plates was perforated and clamped the air cathode (3.8 cm diameter, 7 cm^2 total exposed area), which was treated like previously described for i-MFC. The industrially made anode (20 mm diameter x 30 mm length; 0.18 m^2) consisted of titanium wire connected to a thermally treated graphite fiber brush (fibers of 7.2 μm diameter PANEX33 160K, ZOLTEK, Hungary). Intensity was monitored online as previously described for i-MFC. c-MFC was inoculated by mixing (1:1) fresh medium and the effluent from an already working i-MFC. c-MFC can be further reinoculated to induce a rapid bioaugmentation of the new reactor until the signal response reaches its maximum.

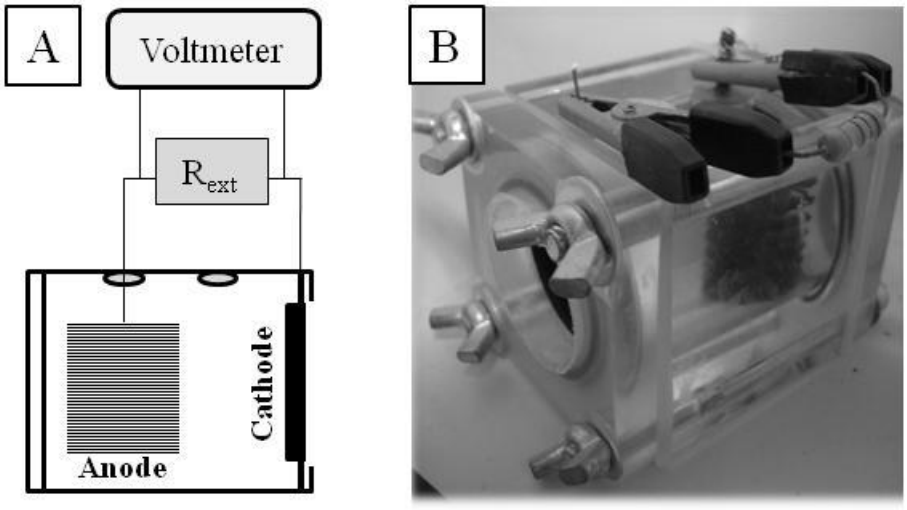

Figure 2.2. Schematic representation (A) and real image (B) of c-MFC

2.3.Cube MEC

An inoculated anode of c-MFCs were transferred to a 32 mL methacrylate vessel with a glass cylinder at the top sealed tightly with a PTFE rubber to build a cube-MEC (c-MEC; figure 2.3). The gas produced was collected in a gas-tight bag connected to the glass cylinder. A constant potential of 0.8V was applied and a 12 Ω external resistance was serially connected to the circuit

to monitor the intensity produced. Before the start of the batch cycles, nitrogen was sparged to ensure anaerobic conditions.

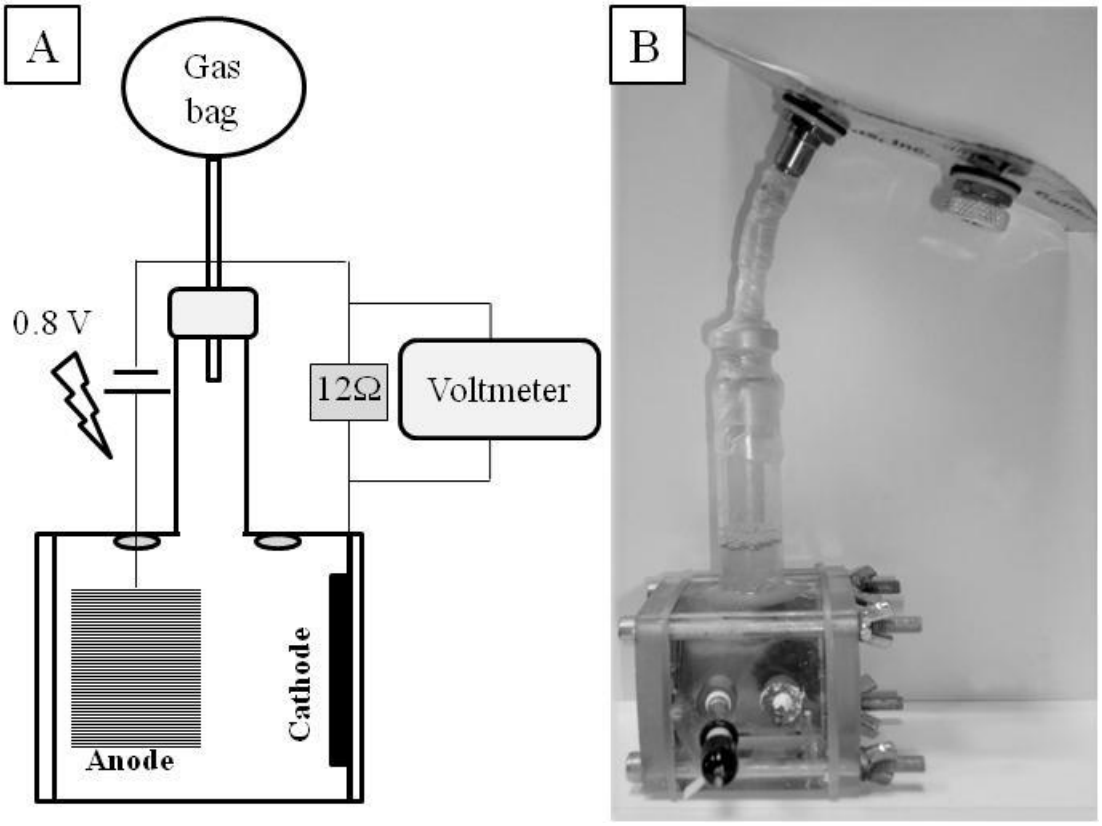

Figure 2.3. Schematic representation (A) and real image (B) of c-MEC

2.4.Medium

The MXCs medium (pH= 7.3) was a 100 mM phosphate buffer solution (PBS; 70 g Na_2HPO_4 and 12 g KH_2PO_4 per liter) with the following components in 1 L of deionized water:

NH_4Cl (0.41 g);

1 mL of 4 g·L-1 $FeCl_2$ stock solution;

0.5 mL of 37.2 g·L-1 $Na_2S·9H_2O$ stock solution.

mineral media (10 mL).

The mineral media had (per liter of deionized water) the composition described in Parameswaran et al. [43]:

EDTA (0.5 g);

$CoCl_2 \cdot 6H_2O$ (0.082 g);

$CaCl_2 \cdot 2H_2O$ (0.114 g);

H_3BO_3 (0.01 g);

$Na_2MoO_4 \cdot 2H_2O$ (0.02 g);

Na_2SeO_3 (0.001 g);

$Na_2WO_4 \cdot 2H_2O$ (0.01 g)

$NiCl_2 \cdot 6H_2O$ (0.02 g)

$MgCl_2$ (1.16 g);

$MnCl_2 \cdot 4H_2O$ (0.59 g);

$ZnCl_2$ (0.05 g);

$CuSO_4 \cdot 5H_2O$ (0.01 g)

$AlK(SO_4)_2$ (0.01 g).

If not different specified (as in chapter 4) 10 mM of BES to MFC and 50 mM to MEC media were added for experiments with methanogenic inhibition.

2.5. Electrochemical calculations

Coulombic Efficiency (CE) was calculated as in equation 2.1.

$$CE = \frac{\text{Coulombs recovered as current intensity}}{\text{Coulombs in substrate}} = \frac{\int_{t_0}^{t_F} I dt}{F \cdot b_{Ac} \cdot V_L \cdot \Delta c \cdot M^{-1}} \qquad (2.1)$$

where t_0 and t_F are the initial and final time of an experiment, Δc is the change in acetate concentration during the experiment (g acetate/L cell), M is the molecular weight of acetate (59 g/mol), b_{Ac} is the number of e^- transferred per mole of acetate (8 mol e^-/mol acetate), F is the

Faraday's constant (96485 C/mol e^-), I is the current intensity and V_L is the volume of liquid in the reactor.

Cathodic gas recovery (r_{CAT}) was calculated as in equation 2.2.

$$r_{CAT} = \frac{\text{Coulombs in } H_2}{\text{Coulombs recovered as current intensity}} = \frac{V_{F,H2} \cdot 2 \cdot F \cdot V_m^{-1}}{\int_{t_0}^{t_F} Idt} \qquad (2.2)$$

where V_m is the molar gas volume (24.03 L/mol) at 20 °C and V_F,H_2 is the volume of H_2 at the end of the cycle.

2.6. Gas chromatography

Volatile fatty acids (VFAs) were analyzed by gas chromatography (Agilent Technologies, 7820-A) using a flame ionization detector (FID) with helium as carrier gas.

H_2 and CH_4 production were analyzed by the same gas chromatograph using a thermal conductivity detector (TCD) with argon as carrier gas. The volumes of hydrogen and methane were calculated using Gas Bag Method [90] and assuming a pressure of 1 atm in the reactor-bag system and room temperature.

CHAPTER 3

DNA-based analysis of the microbial community structure in single-chamber MXCs

Motivation

In bioelectrochemical studies is of high importance to understand ARB optimal growth conditions to ensure the maximal performance of MXCs. In that direction, DNA-based microbiological analysis may help to solve this important point.

Among the many different factors, from cell configuration to medium composition, that have been studied so far affecting MFC performance [91–93], the role of external resistance has gained a lot of interest due to its significant effect on biofilm performance and characteristics [94–97]. These works show how the external resistance influences the power output in MFC. The choice of an external load equal to the internal cell resistance leads to an optimum MFC operation. On the other hand, the external resistance indirectly poises an anode potential, a key parameter in bioelectrochemical systems, since it determines the energy gain for the bacteria, and thus enhances the growth of different anode microbial populations [94,96] and biofilms with different morphologies [98]. While the effect of external resistance on MFC performance is already described, the response of microbial communities grown with different external resistances when operated in MEC is yet to be studied.

The aim of this chapter was the development of the tools required for using quantitative real time-polimerase chain reaction (qPCR) in MXCs (section 3.1). qPCR technique was applied to characterise the microbial communities of single-chamber MFCs obtained using different external resistances. Thus, the work presented in section 3.2 aims at investigating whether bioanodes selected at different external resistances under MFC operation present different responses under MEC operation.

3.1. Quantitative real-time PCR (qPCR)

The work presented in this section was developed in collaboration with the Department of Genetics and Microbiology at Universitat Autònoma de Barcelona and with the help of Dr. M. Pilar Cortés

3.1.1. qPCR standard curves generation procedure

Amplification of the 16S rRNA gene with conventional PCR technique

Genomic DNA of *Geobacter sulfurreducens* (*G. sulfurreducens*, DSM 12127T) and *Methanobacterium formicicum* (*M. formicicum*, DSM 1535T) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) to construct the standard curves. *G. sulfurreducens* was used as standard for Fe(III)-reducing *Geobacteraceae* family, and *M. formicicum* was used as standard for total *Achaea* and order *Methanobacteriales*. Specific primers (Table 3.1) were used to amplify the V5-V6 region from *M. formicicum* and the 16S V3-V4 region of *G. sulfurreducens* [99].

Conventional PCR was done in a final volume of 100 μl using the following final concentrations: 1.5 mM of Buffer; 0.2 mM of each dNTP); 0.5 mM of each specific primer; and 5.6 U/μL of volume activity of Taq-polymerase (Expand High Fidelity PCR system; Roche Diagnostics); 100 ng/μl DNA-template and sterile double-distilled water up to 100 μl.

The temperature profiles for PCR reactions were as follows: initial denaturation of 5 min at 95 °C; 30 cycles of 30' at 95 °C, 30' at 55 °C for *Geobacter* primers and DNA-template and 30' at 60 °C for *Methanobacteriales* primers and DNA-template and 45' at 72 °C; final elongation at 72 °C for 7 min and cooling to 4 °C. PCR products were gel-purified using GFX PCR DNA & Gel Band purification kit (Healthcare), according to the manufacturer's instruction, and dialyzed.

Table 3.1. Conventional PCR data for each specific target.

Target	DNA-template	Primers	Annealing temperature	Amplicon size
Archaea	*M. formicicum*	ARC787F ARC1059R	55ºC	271 bp
Methanobacteriales	*M. formicicum*	MBT857F MBT1196R	55ºC	342 bp
Geobacter	*G. sulfurreducens*	GEO561F GEO825R	60ºC	265 bp
Archaea - Methanobacteriales	*M. formicicum*	ARC787F MBT1196R	55ºC	416 bp

F: Forward primer; R: Reverse primer

Ligation and transformation of the 16S rRNA gene fragments

The purified and dialyzed 16S rRNA gene amplicons were cloned into the pGEM®-T Vector System, according with the manufacturer's protocol (Promega, Mannheim, Germany). After a successful insertion of the 16S rRNA gene fragments into the pGEM®-T vectors (Figure 3.1), they were transformed by using electroporation procedure into high efficient electrocompetent cells of Escherichia coli DH5α (Invitrogen).

100 μL of each transformation culture was plated out onto Lysogeny broth (LB) agar plates with 50 μg/μL of ampicillin and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). Afterwards the plates were incubated for 24 h at 37°C. Positive clones were picked and cultured overnight in 5 ml LB broth added with 50 μg/μL of ampicillin.

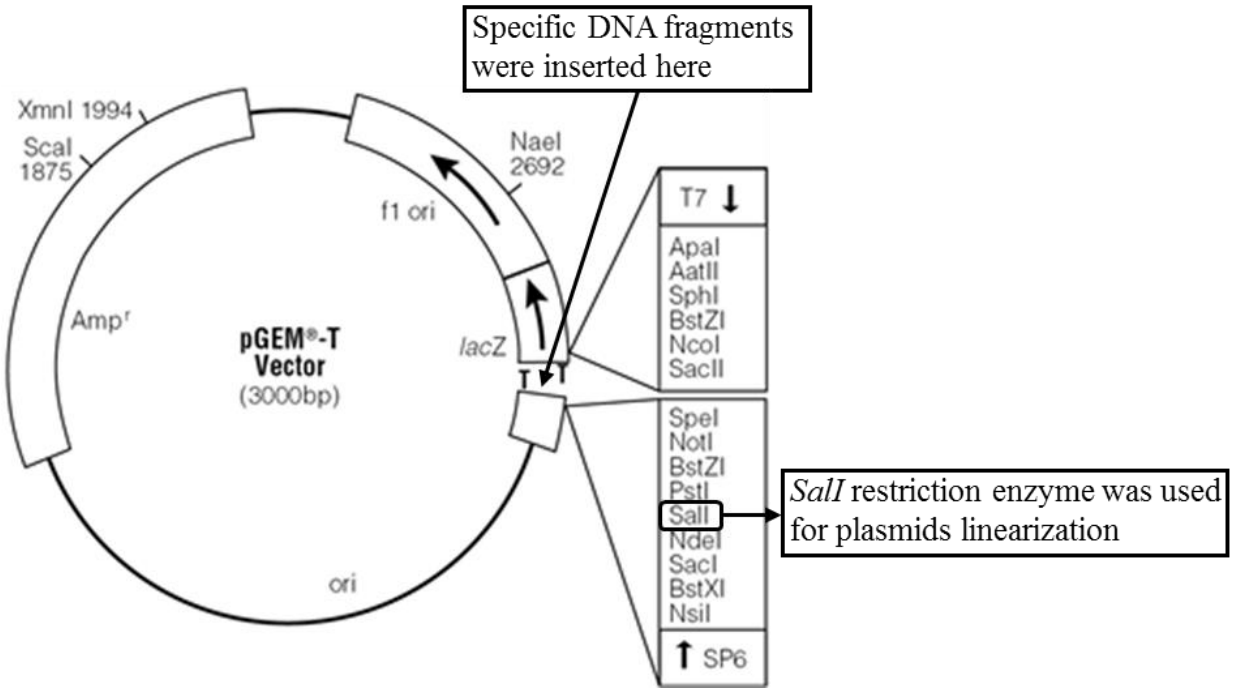

Figure 3.1. Schematic representation of pGEM®-T Vector (Promega).

Plasmid isolation

GeneJETTM Plasmid Miniprep Kit (Fermentas, Life Sciences) was used for plasmid isolation, according to manufacturer's instructions. To verify if the plasmids had inserted the right 16S rRNA gene fragment, conventional PCR was performed by using the same PCR mixture composition and PCR temperatures described before. Primers couples used were: M13F and ARC1059R (annealing temperature 55 ºC); M13F and MBT1196R (annealing temperature 55 ºC); M13F (CGCCAGGGTTTTCCCAGTCACGAC) and GEO825R (annealing temperature 60 ºC). A 1.5% agarose gel electrophoresis was carried out to check the size of PCR products. Sequences of the respective cloned fragments were confirmed by sequencing (Macrogen) and then comparing with the BLAST database of the NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Plasmids which had inserted correctly the 16S rRNA gene fragment were stored at –80 °C.

Linearization of the plasmids for qPCR standard curves

Afterwards, plasmids containing target fragments were linearized using SalI restriction enzyme (New England Biolabs) according to manufacturer's instructions (Figure 3.1). A 1% agarose gel electrophoresis was carried out by using 100 µL of linearization product. DNA was recovered from agarose gel using GFX PCR DNA & Gel Band purification kit (Healthcare), according to manufacturer's instructions, and dialyzed.

qPCR protocol

LightCycler® 480 System was used to perform the qPCR in LightCycler® 480 multiwell plate 96 white (Roche Diagnostics).

qPCR was performed using the corresponding primers and probes previously described [100–102] (Table 3.2). Each reaction mixture of 20 µL was prepared using the LightCycler 480 Probe Master kit (Roche Diagnostics), primers for *Archaea* and *Methanobacteriales* (final concentration 500 nM), primers for *Geobacter* (final concentration 300 nM), hydrolysis probes for *Archaea* and Methanobacteriales (final concentration 200 nM), hydrolysis probes for *Geobacter* (final concentration 100 nM), 2X LC480 Probe Master and 2 µl of template DNA.

Amplification of *Archaea* and *Methanobacteriales* was performed as described by Yu et al. [100] with slight modifications. Reactions were performed in a three-step procedure: denaturation for 10 min at 94 °C followed by 45 cycles of 10 s at 94 °C, 40 s at 60 °C and 1 s at 72 °C (fluorescence detection). Similarly, *Geobacter* was quantified as described in the literature [101,102] with a small modification: denaturation for 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 60 s at 55 °C and 1 s at 72 °C (fluorescence detection). All DNA templates were analyzed in duplicate.

Table 3.2. Primers and probes used for qPCR.

Target	Primer/probes set	Sequence (5'-3')	Reference
Archaea	ARC787F	ATTAGATACCCSBGTAGTCC	[100]
	ARC1059R	GCCATGCACCWCCTCT	
	ARC915 probe	AGGAATTGGCGGGGGAGCAC	
Methanobacteriales	MBT857F	CGWAGGGAAGCTGTTAAGT	[100]
	MBT1196R	TACCGTCGTCCATCCTT	
	MBT929 probe	AGCACCACAACGCGTGGA	
Geobacter	GEO561F	GCCATGCACCWCCTCT	[101,102]
	GEO825R	GCGTGTAGGCGGTTTCTTAA	
	Gbc1 probe	AGCACCACAACGCGTGGA	

F: Forward primer; R: Reverse primer

qPCR calibration curves construction and calculation of the limits of detection and quantification

Each plasmid was serially diluted with a 10-fold step ranging from 10^2 to 10^7 copies per PCR reaction mix and directly used as template for qPCR with the corresponding primers and probes (Table 3.2). The number of gene copies was calculated converting the DNA concentration [ng/µL] by using equation 3.1 as previously described [103]:

$$\text{gene copies} = \left(\text{DNA concentration } \left[\frac{\text{ng}}{\mu\text{L}}\right]\right) \cdot \left(\frac{1\text{ g}}{10^9\text{ ng}}\right) \cdot \left(\frac{1\text{ mol bp DNA}}{660\text{ g DNA}}\right) \cdot \left(\frac{6.023 \cdot 10^{23}\text{ bp}}{\text{mol bp}}\right) \cdot \left(\frac{1\text{ copy}}{\text{plasmide size [bp]}}\right) \cdot \text{volume of template [µL]} \quad (3.1)$$

where the plasmid size depends of pGEM®-T vector size (3000 bp) and of the size of the specifics amplicons obtained by using conventional PCR (Table 3.1).

The quantification cycle (Cq) values determined were plotted against the logarithm of their initial template copy numbers. Each standard curve was conducted in triplicate.

The number of 16S rDNA copies in plasmid DNA was calculated using the previously described equation [103]. Finally, 16S rDNA concentration (copies/mg) of *Geobacter*, *Methanobacteriales* and *Archaea* in the different samples studied was obtained using equation 3.2.

$$\text{nº copies per mg} = \frac{\text{elution volume [µL]}\cdot\text{concentration extracted DNA}\left[\frac{\text{ng}}{\mu\text{L}}\right]}{\text{mg of sample}} \cdot \frac{\text{nº copies per reaction mix}}{\text{concentration template DNA}\left[\frac{\text{ng}}{\mu\text{L}}\right]\cdot 2\ \mu\text{L}} \qquad (3.2)$$

3.1.2. Obtaining qPCR standard curves

qPCR standard curves obtained with no-linearized plasmids

Across the procedure previously described, several problems were detected and finally solved to obtain three standard curves.

The first setting of the standard curves generation conteplated the use of three no-linearized plasmids with a linear range of detection spanning eight orders of magnitude from 10^1 to 10^8 copies per reaction mix. Three standard curves were generated in triplicate in each assay by using three different primers and probe sets as described in Table 3.2.

As the curves obtained were not reproducible with tolerable standard deviation, several assays were repeated to ensure that the error was not due to the user experimental action. Two main problems were detected by using these curves:

i) *Methanobacteriales* quantification was higher than total *Archaea* quantification which was unrealistic

ii) The error (n=3) was higher than 0.02 which is the limit recommended for LC480.

Generation of Archaea-Methanobacteriales "mixed" plasmid containing the two specific 16S rDNA

The same plasmid was used to build simultaneous *Methanobacteriales* and *Archaea* standard curves in order to reduce the margin of experimental error and simplify the experimental procedure. A bigger sequence of 416 bp was isolated from genomic DNA of *M. formicicum* DNA template with conventional PCR by using ARC787F primer and MBT1196R primer (see Figure 3.2). This sequence, which includes the two specific 16S rDNA regions described by Yu et al. [100], was cloned as previously described and the linearized plasmid was used to generate *Methanobacteriales* and *Archaea* standard curves.

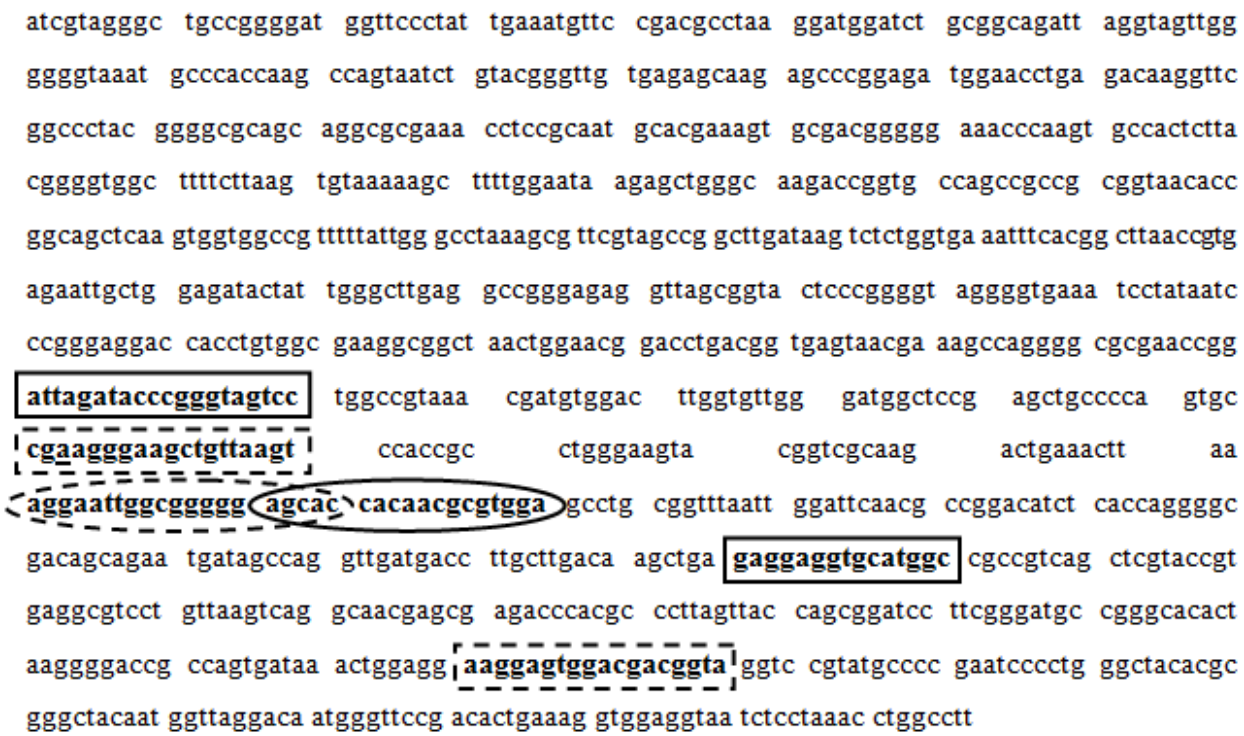

Figure 3.2. *M. formicicum* 16S rRNA gene sequence obtained from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/M36508.1). Sequences which are targets for total *Archaea* (solid line) and *Methanobacteriales* (dotted line) primers (squares) and probes (ellipses) were pointed out in this figure.

Three qPCR standard curves obtained with two linearized plasmids

The previously described plasmids linearization step was added to the protocol in order to obtain repetitive standard curves. Three standard curves were obtained by using two plasmids containing 16S rDNA specific fragments cloned (Figure 3.3).

The curves obtained with the linearized plasmids had an error lower than 0.02 (n=3), with a linear range of detection spanning six orders of magnitude from 10^2 to 10^7 copies per reaction mix. A reproducible detection limit of 100 target copies was established in all the cases. The curves represented in figure 3.3 were finally used as standard in sections 3.2 and 4.1.

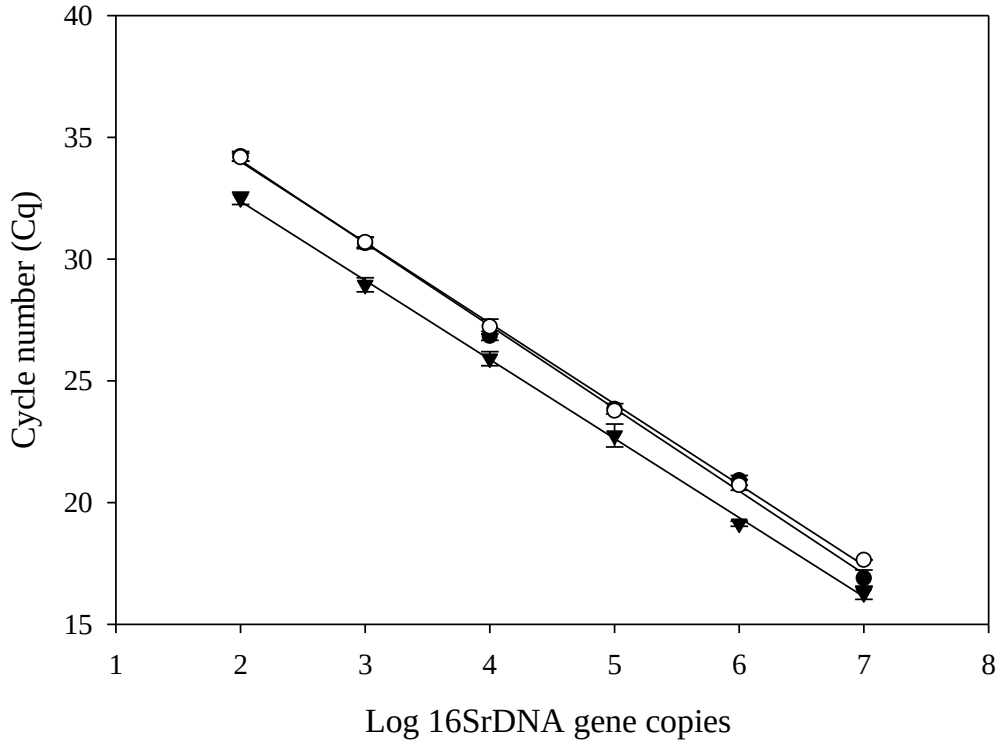

Figure 3.3. Standard curves for 16S rDNA. The standards curves were constructed by using linear plasmid DNA and amplifying the corresponding target of -○- *Geobacter*, -●- *Methanobacteriales* and -▼- *Archaea*. Values represent means (n=3) ± standard errors or deviation.

3.2. Use of qPCR to evaluate the effect of different external resistances

Part of the content of this section has been submitted for publication to Water Science and Technology as: L. Rago, J. A. Baeza, A. Guisasola. Performance of microbial electrolysis cells with bioanodes grown at different external resistances

3.2.1. Specific experimental procedures for section 3.2

MFC and MEC operation

The anodes of three c-MFCs were inoculated by mixing (in a volume ratio 1:1) fresh medium and media from an already working i-MFC (with an external resistance of 12 Ω). For each c-MFC, the external resistance to connect both electrodes was different: 12 Ω (MFC_{12}), 220 Ω (MFC_{220}) and 1000 Ω (MFC_{1000}).

The MFCs achieved a stable operation after around 60 d, when similar performance was observed in consecutive cycles. Subsequently, the configuration was changed to MEC by closing the lateral aperture and hence preventing oxygen from entering to the cathode. An external potential (0.8 V) was supplied to drive the cathodic hydrogen production. All MFCs and MECs were operated in batch mode. The fresh medium was the 100 mM PBS medium prepared as explained in chapter 2 and fed with 30 mM of acetate.

Electrochemical analysis

Chronoamperometric analyses were performed to evaluate MEC performance using a Multi Autolab system (Ecochemie, Utrecht, Netherlands). The anode was set as the working electrode and the cathode was used as both the auxiliary and the reference electrode. The anode potential

(vs. cathode potential) was set at 13 levels ranging between 250 mV and 850 mV in steps of 50 mV. Each potential was set for 300 seconds to allow current intensity to stabilize. The last data point corresponding to each potential was used to build the curve Current intensity vs. Electrode potential.

Similar chronoamperometric measurements were used to build polarization and power curves for MFCs, where current intensity vs. cell potential and power vs. current intensity were respectively evaluated. In this case, the anode potential (vs. cathode potential) was set at 17 levels from -675 mV (anode open circuit potential) to -20 mV. The applied potentials were negative, because it is equivalent to using different external resistances. Finally, power curves were calculated from the polarization curves as the product between potential and intensity.

DNA extraction

Total DNA was extracted from approximately 0.15 g of samples using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The Inoculum sample was obtained centrifuging at 10000 g (Beckmann Coulter TM, Avanti J20XP; USA) 350 ml of the media obtained from the running *Inoculum*-MFC, which was used to inoculate the cube MFCs ($MFC_{12,}$ MFC_{220}, MFC_{1000}). The MFC samples (after 60 days of operation) and the MEC samples (after 30 days of operation) were obtained from the anodes of each cell. The anode graphite fibers were cut and combined for DNA extraction. Previously, the fibers were rinsed with 1 ml of sterile MilliQ water to remove residues from the growth medium or residues from biofilm. Quality and quantity of the DNA was measured using a NanoDrop® spectrophotometer (ThermoScientific). Moreover, DNA was visualized under UV in a 0.7% gel electrophoresis with TBE 0.5X (Tris-Borate 50 mM; EDTA 0.1 mM; pH 7.5-8).

3.2.2. Results and discussion

MFC operation with different external resistances

The three c-MFCs were inoculated with the same inoculum from an i-MFC. Part of the i-MFC medium (350 ml) was sampled for DNA extraction. After inoculation, each MFC was operated in batch cycles (5 days per cycle) using a different external resistance: 12 Ω (MFC_{12}), 220 Ω (MFC_{220}) and 1000 Ω (MFC_{1000}). Current density and power values obtained from the first five batch cycles for the three MFCs are shown in Figure 3.4.

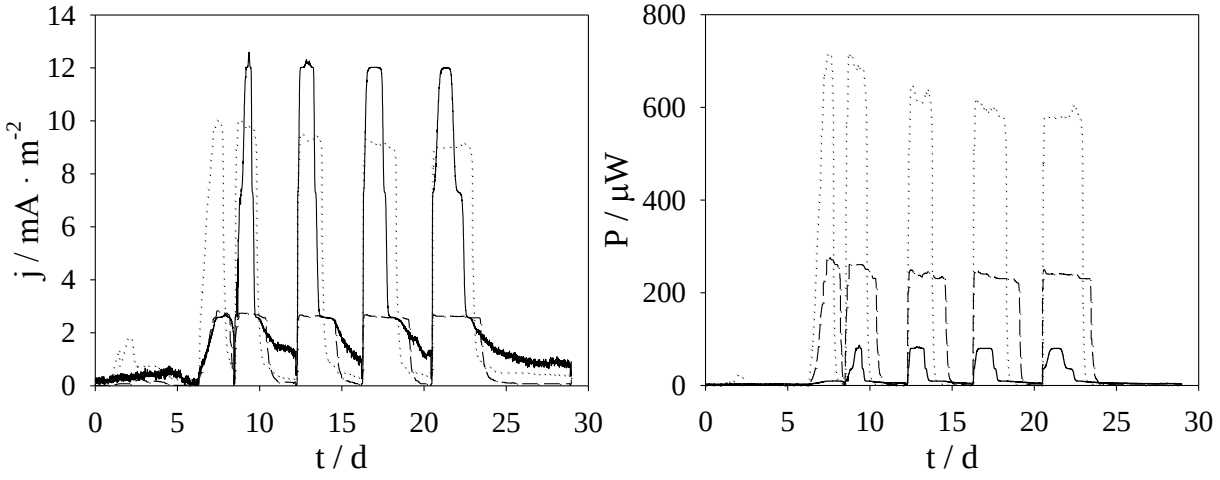

Figure 3.4. Evolution of current density (left) and power output (right) along time in three MFCs with different external resistance (MFC_{12}-solid; MFC_{220}-dotted; MFC_{1000}-dashed).

A higher current density was obtained by using a lower external resistance in agreement with previous literature reports [94,95]. Coulombic efficiency (Coulombs recovered as current intensity / Coulombs in substrate) also showed a trend versus external resistance resulting in a higher value for lower external resistances: MFC_{12} (74 %), MFC_{220} (53 %) and MFC_{1000} (23 %). However, despite its high current density, the lowest power output was obtained in MFC_{12}. This controversial observation was previously discussed in the literature. Zhang et al. [98] stated that

this lower power output might be due to the significant ohmic losses resulting from void spaces in the interior of the biofilm. The use of an external resistance lower than the optimum value generates a reduction of the electrical conductivity within the biofilm which has a higher active biomass but also a higher exopolysaccharide content. On the other hand, the highest power output was obtained from MFC_{220} (MFC_{220}: 718μW, MFC_{12}: 86μW and MFC_{1000}: 279μW) due to the closeness between the external resistance and the cell internal resistance. The internal resistance of the MFCs used in this work was estimated to be 150 Ω through polarization curves (data not shown). The experimental data in this work corroborates that an optimal external resistance has to be used to obtain higher maximum power output.

Transference of MFC anodes to MEC

After 60 days of MFC operation, under steady-state MFC operation, the anodes of MFC_{12}, MFC_{220} and MFC_{1000} were sampled for DNA extraction and transferred to three different MECs (MEC_{12}, MEC_{220} and MEC_{1000}) with 0.8 V of applied voltage between anode and cathode. The current density values obtained from the three MECs during four weeks are shown in Figure 3.5. The anodes obtained from MFC_{12} and MFC_{220} (i.e. inoculated with lower external resistances) were immediately adapted to the new configuration; indeed, the maximum current density was achieved from the first cycle in MEC_{12} and from the second one in MEC_{220}. On the other hand, the anode from MFC_{1000}, inoculated with the higher external resistance, needed more than one week (5 batch cycles) to achieve its best performance. Although the initial current density produced by MEC_{12} was the highest, it decreased around 15% throughout the 25 days of MEC operation, whereas the current density produced by MEC_{220} and MEC_{1000} did not decrease after the start-up period. Despite the 15% decrease in current intensity for MEC_{12}, this cell provided the best performance and faster adaptation to the MEC operation. The volume of hydrogen produced was measured for each MEC at the end of each of the last two batch cycles. Hydrogen production rate in MEC_{12} (1.54 ± 0.13 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$, n=2) was slightly higher than those in MEC_{220} (1.34 ± 0.02 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$, n=2) and MEC_{1000} (1.1 ± 0.3 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$, n=2) in agreement with the trend in intensity values. Moreover, in all the cases, the coulombic efficiency was around 90%.

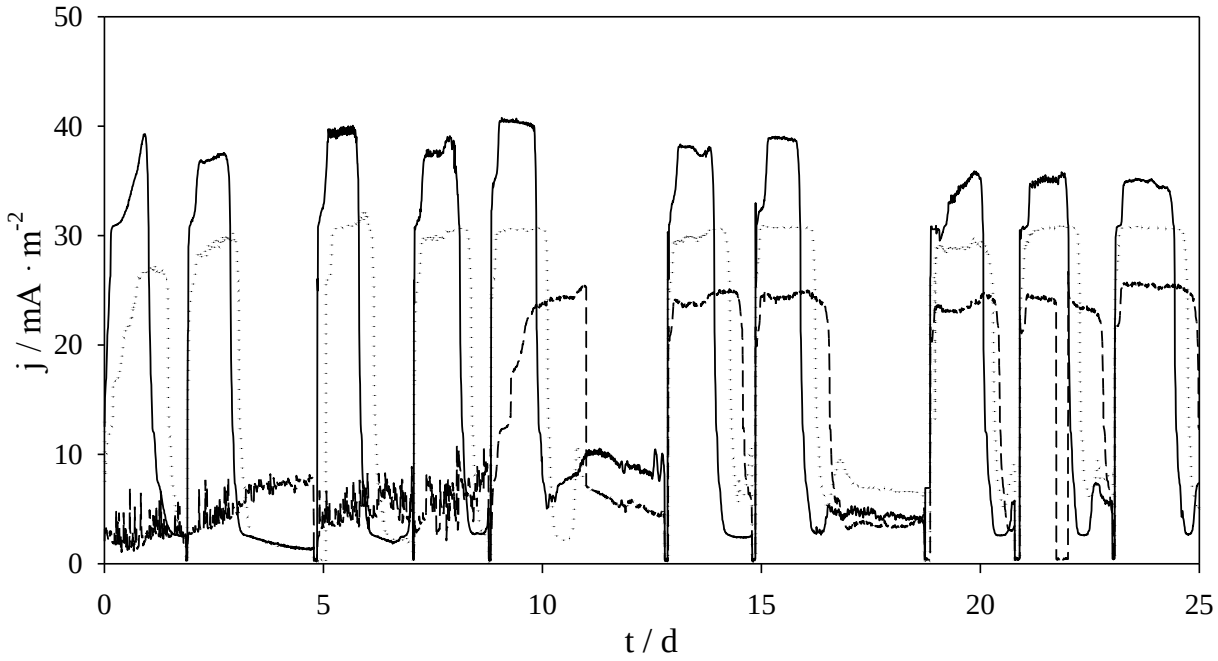

Figure 3.5. Current intensity versus time measured in the three MECs (MEC_{12}-solid; MEC_{220}-dotted; MEC_{1000}-dashed).

Figure 3.6 shows the chronoamperometric measurements from the three MECs after four weeks of operation. For a certain applied potential, higher intensity values were obtained from the anode operated with lower external resistance in MFC. For example, at an applied potential of 0.8 V, the highest current intensity was obtained from MEC_{12} (43 mA · m^{-2}) and from MEC_{220} (35 mA · m^{-2}), while in MEC_{1000} (25 mA · m^{-2}) it was 41% lower than that obtained for MEC_{12}. After 30 days, DNA was extracted from anodic samples for qPCR analysis.

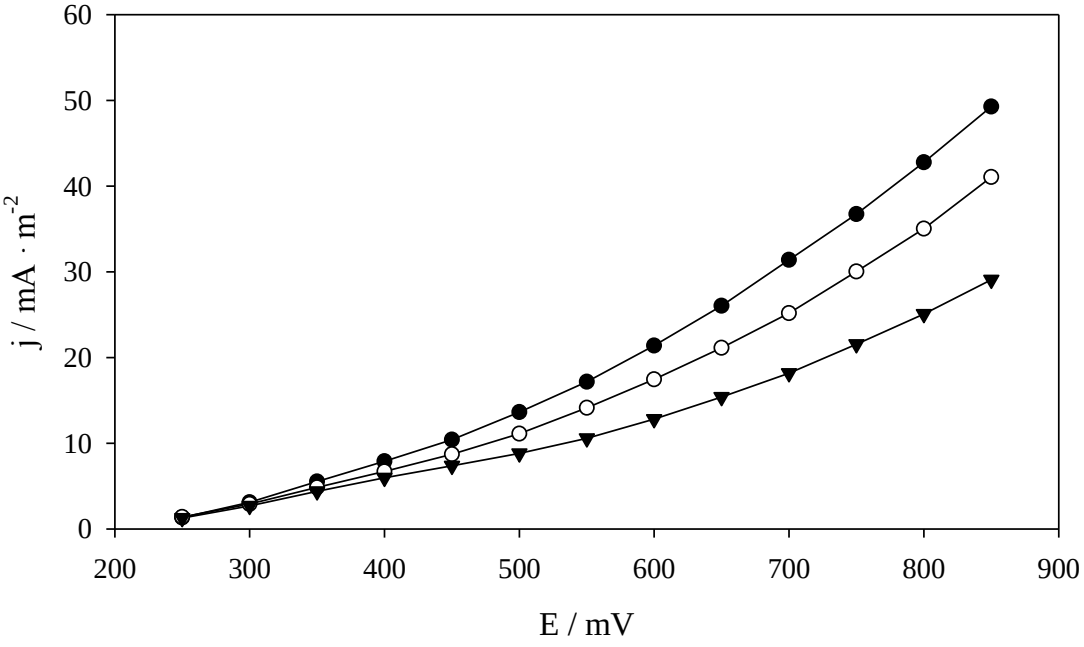

Figure 3.6. Chronoamperometric measurements obtained by setting the anode potential in the range 250 - 850 mV in the three MECs (—●— MEC_{12}; —○— MEC_{220}; —▼— MEC_{1000}).

The DNA extracted from inoculum, MFCs and MECs samples was quantificated by qPCR (Figure 3.7), using the qPCR curves obtained in section 3.1. The concentration of planktonic *Geobacter* in the inoculum was 6.98 x 10^5 gene copies·mg^{-1}. Concentration of *Geobacter* in the anode increased in MFCs and was further augmented in MECs. The presence of *Geobacter* was similar for MFC_{12} (1.69 x 10^7 gene copies·mg^{-1}) and MFC_{220} (1.17 x 10^7 gene copies·mg^{-1}) anodic biofilms, but only 1.48 x 10^6 gene copies·mg^{-1} were obtained from MFC_{1000}. Planktonic *Geobacter* concentration in the inoculum sample was lower compared with anodic biofilms samples obtained from the MFCs (around 20 times in the case of MFC_{12} and MFC_{220}, and two times for MFC_{1000}). Then, the development of a biofilm with a high content of *Geobacter* was possible in all the anodes, although the best development was demonstrated for external resistances of 12 and 220 Ω. In addition, the higher *Geobacter* concentration in MFC_{12} and MFC_{220} anodic samples justifies the CE values, which were higher for these reactors than for MFC_{1000}. The amount of *Geobacter* in MECs was higher than in MFCs. It was not detected a clear trend among MEC anodic biofilm samples in contrast to MFCs: after four weeks of

operation, all MECs had almost the same amount of *Geobacter* between 4.38 x 10^7 (MEC_{12}) and 4.92 x 10^7 gene copies·mg^{-1} (MEC_{1000}), regardless of the external resistance used in MFC operation. Furthermore, it has been demonstrated that the applied potential and the anoxic conditions of MEC reactors favored *Geobacter* growth in the anodic biofilm, which resulted in a concentration more than double with respect to the MFCs anodic biofilm. The improved operational conditions with respect to MFC (total absence of oxygen and applied voltage) also justified the increased CE around 90% in all three MECs.

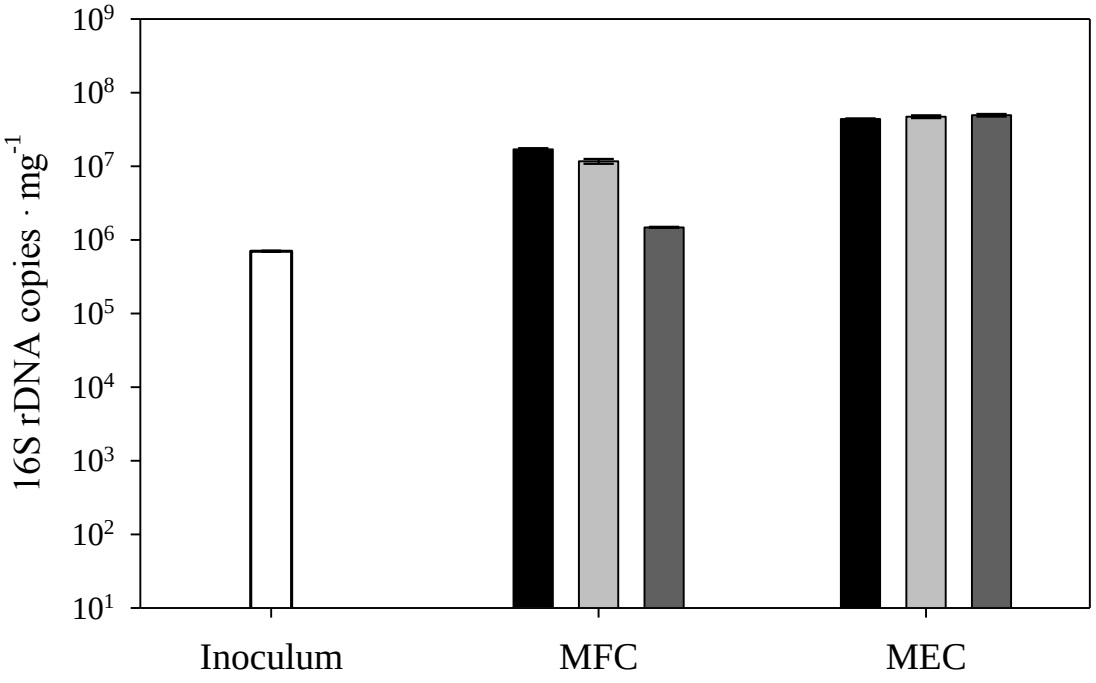

Figure 3.7. qPRC results of *Geobacter* (mean of triplicate values ± standard deviation) in the inoculum and anodes of MFC and MEC. Black color: 12 Ω; light grey: 220 Ω; dark grey: 1000 Ω.

Considering all the results presented, this work shows that the shortest start-up for an MEC is obtained using a very low external resistance (12 Ω). Although the power output in MFC_{12} is the lowest observed (Figure 3.4 right), it provides the highest current intensity under MFC operation

(Figure 3.4 left), which is the key parameter related to *Geobacter* activity and hence it provides the best development in the anodic biofilm (Figure 3.7). This allows a very fast adaptation to MEC operation: in just one cycle MEC_{12} is able to achieve a current intensity very close to its maximum value. In addition, after the start-up period, MEC_{12} achieves the highest current intensity compared to MEC_{220} and MEC_{1000} (Figure 3.5) and also the maximum current density at different applied potentials (Figure 3.6).

The utilization of a 220 Ω resistance also provides good performance, although current intensities are slightly lower both in MFC and MEC. High resistances as 1000 Ω are not recommended because *Geobacter* growth is more limited (lower current intensity), which leads to a slower start-up under MEC operation. Nevertheless, if the start-up period is not a key problem, any of the three resistances allows developing an anodic biofilm with a high content in *Geobacter* near 5×10^7 gene copies·mg^{-1}.

3.3.Conclusions

Three qPCR stardard curves were developed for absolute quantification of *Archaea*, *Methanobacteriales* and *Geobacter*. The curves obtained with linearized plasmids had a really low error (lower than 0.02) and the reproducible detection limits were between 100 and 10^7 target copies in all the cases. Thus, qPCR technique was confirmed as a good working tool for quantifying *Archaea*, *Methanobacteriales* and *Geobacter* in reactor samples. Moreover, qPCR standard curves permits to describe the quantitative composition of the *Methanobacteriales* versus all *Archaea*.

qPCR *Geobacter* stardard curve was used to analyze the samples obtained from the reactors built to the study of external resistances effect in section 3.2. In this section, an anode inoculated under low external resistance in MFC mode (12 Ω) showed better performance in the posterior MEC mode (i.e. gave higher current intensity and showed higher H_2 production rate) than other MFCs inoculated under higher resistances (220 and 1000 Ω). qPCR confirmed that the use of a low external resistance provides an MFC anodic biofilm with the highest content of *Geobacter*, because a low resistance allows higher current intensity, which is correlated to exoelectrogenic

activity. The lowest resistance tested also allowed the faster transition to stable MEC operation and lead to an anodic biofilm able to drive higher current intensities. However, long-term MEC operation allowed developing microbial anodic biofilms with similar *Geobacter* content around 5 x 10^7 gene copies·mg^{-1} in spite of the initial differences after MFC operation with different resistances. Moreover, the use of a not too low external resistance, closer to the internal MFC resistance, is recommended to obtain a higher power in MFC.

CHAPTER 4

Limitations of the long-term utilization of 2-bromoethanesulfonate as methanogenic inhibitor in bioelectrochemical systems

Motivation

Methanogenesis is one of the major drawbacks in the full-scale application of bioelectrochemical systems. Methanogenesis suppression is achieved, at lab-scale, with the use of chemical inhibitors such as 2-bromothanesulfonate (BES) or lumazine. BES is the most reported chemical inhibitor for methanogenesis in bioelectrochemical systems at lab-scale. As previously described in chapter 1, this option has been widely accepted in the literature despite its economic cost makes it unrealistic for full-scale systems.

The origin of this study is an unexpected methane production in a system amended with BES (with a concentration in the range of those reported in the literature) [55]. This observed methane production casts doubts on the long-term effectiveness of this compound in MXCs. As one of the hypotheses presented to explain this observation, potential BES degradation was studied.

Therefore, the aim of this chapter was to evaluate the extent of BES effect on MEC performance (section 4.1) and its potential degradation in different bioelectrochemical systems (section 4.2).

4.1. Microbial community analysis in a long-term membrane-less microbial electrolysis cell with hydrogen and methane production

Part of this section was published in Bioelectrochemistry as: L. Rago, Yolanda Ruiz, J. A. Baeza, A. Guisasola, P. Cortés (2015), Microbial community analysis in a long-term membrane-less microbial electrolysis cell with hydrogen and methane production. Bioelectrochemistry. [49].

4.1.1. Specific experimental procedures for section 4.1

The concentric MEC

A large single-chamber membrane-less MEC (1300 mL, Figure 4.1) was used to carry out all the experiments presented in this section and in section 4.2.

The anode for the 1.3L MEC was a graphite handmade brush, made as described before (chapter 2), previously inoculated in an i-MFC. The cathode (A=0.034 m^2) was made with carbon cloth coated with carbon powder and platinum suspension on the side facing the anode. Both electrodes were arranged concentrically with the cathode in the outer perimeter, so that all ends of the anode were at the same distance from the cathode. The gas produced was collected in a gas-tight bag connected to the head-space of the reactor. A valve in the bottom part of the MEC permitted liquid sampling and nitrogen sparging to ensure anaerobic conditions before the start of the batch cycle.

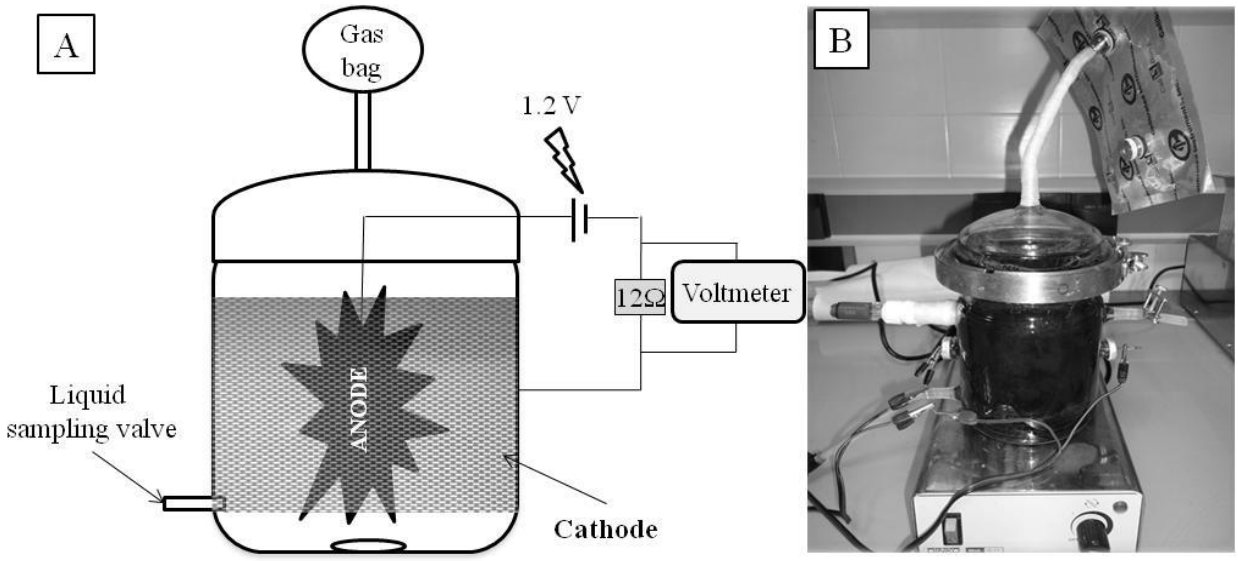

Figure 4.1. Schematic representation (A) and a real image (B) of the 1.3L concentric MEC

The reactor was operated in batch mode, with constant agitation and an applied voltage of 1.2 V (HQ Power, PS-23023). The intensity produced was monitored online as previously described for cube MEC.

Experimental setup

In this study, three experimental periods were conducted by using different BES concentration: 50 mM, 100 mM and 200 mM. The MEC was inoculated and operated with acetate as substrate (12 mM) for 4 months using 50 mM of BES concentration, according with common practice [55]. Afterwards, the medium was replaced twice adding increasing BES concentrations. First, 100 mM of BES concentration was maintained for 15 days (one batch cycle) and finally fresh medium with 200 mM of BES was added and operated for 20 additional days (one interrupted batch cycle).

Chemical analysis

CH_4 relative composition was calculated as the ratio of CH_4 with respect the total amount of CH_4 and H_2 and was calculated as in equation 4.1.

$$\text{Relative composition } CH_4 = \frac{\text{Volume } CH_4}{\text{Volume } H_2 + \text{Volume } CH_4} \qquad (4.1)$$

DNA extraction

DNA samples were obtained at the end of each period from anode and cathode. Medium sample was collected collected from 1.3 L MEC only at the end of the period with 50 mM of BES concentration. The anode graphite fibers were rinsed with 1 ml of sterile MilliQ water to remove residues from the growth medium or residues from biofilm and then were cut and combined for DNA extraction. The same process was applied for the cathode carbon cloth. The medium was sampled 1.3 L of the MEC medium was centrifuged at 10000g (Beckmann Coulter TM, Avanti J20XP; USA) to remove supernatant. The pellet obtained was used for DNA extraction.

Total DNA was extracted from approximately 0.15 g of wet samples using a PowerSoil DNA Isolation Kit according to the manufacturer's instructions. Quality and quantity of the DNA was measured using a NanoDrop® spectrophotometer (ThermoScientific). Moreover, DNA was visualized under UV in a 0.7% gel electrophoresis with TBE 0.5X (Tris-Borate 50 mM; EDTA 0.1 mM; pH 7.5-8).

High-throughput 16S rRNA gene pyrosequencing

Pyrosequencing was performed in a 454 Titanium FLX system by the Research and Testing Laboratory (RTL, Lubbock, TX) based upon RTL protocols from DNA samples (20 ng· μL^{-1}, quality ratio of 1.8).

DNA samples were with an average of 3000 reads/assay by using different couples of primers of the bacterial and *Achaea* 16S rRNA gene:

- 338F-907R which comprising the V3-V5 regions of the bacterial 16S rRNA gene [104];
- 349F-806R that comprising the V3-V4 regions of the *Archaea* 16S rRNA gene [69,105]

Sequences were checked using Dechipher (Database Enabled Code for ideal Probe Hybridization Employing R) with Decipher's Find Chimeras web tool to uncover short-length sequence (less than 1000 nucleotides) chimeras (http://decipher.cee.wisc.edu/FindChimeras.html [82]).

Sorting and trimming were carried out using the Pipeline Initial Process at the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://rdp.cme.msu.edu/index.jsp [77] with the default settings. The RDP Classifier was used to assign 16S rRNA gene sequences to a taxonomical hierarchy with a confidence threshold of 95%, since the DNA sequences were <250 bp [106]. The relative abundance of a given phylogenetic group was calculated as the number of sequences associated with that group divided by the total number of sequences per sample.

Quantitative real-time PCR

Quantitative hydrolysis probes based real-time PCR (qPCR) was used to quantify total *Archaea*, the hydrogenotrophic methanogen order *Methanobacteriales* (MBT) and the exoelectrogen proteobacteria *Geobacter* as a member of the Fe(III)-reducing *Geobacteraceae* family.

qPCR was performed with a Lightcycler 480 instrument (LC480; Roche) using the corresponding primers and probes as previously described in Section 3.1.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to analyze the samples of graphite fiber brush (anode) and carbon cloth (cathode) collected from 1.3 L MEC when it was working with 50 mM of BES concentration. Samples were fixed with a solution of 2.5% glutaraldehyde (Sigma-Aldrich) and 2% paraformaldehyde (Sigma-Aldrich), and were processed according to conventional electron microscopy methods as previously described [107]. Samples were treated

with osmium tetraoxide, dehydrated with ethanol and dried at critical point with carbon dioxide (BAL-TEC CPD030; Bal-Tec). Then, the samples were coated with few nanometers of Au-C (E5000 Sputter Coater) to increase signal detection and visualized on a Scanning Electron Microscope (Hitachi S-70). As a control, unused fiber brush and carbon cloth were examined following a similar procedure.

4.1.2. Results

Exploring CH_4 sources in a BES-fed membrane-less MEC

A 1.3L MEC was inoculated using an anode from an operating i-MFC and an abiotic cathode. The MEC was inoculated and operated for 4 months with a BES concentration of 50 mM, enough to avoid methanogenesis according to common practices [55]. In spite of this, CH_4 was produced. According to previous studies [50], methane production could be due to the activity of methanogenic *Archaea*, attached either to the anode, to the cathode or suspended in the cell medium (including those attached in the cell walls). A simple experiment was conducted in open circuit as a first assessment of the CH_4 source (figure 4.2). The operating anode and cathode were separated into two different cells without the complementary electrode. A new abiotic cathode was added to the operating anode and a new abiotic anode was added to the operating cathode. Two different batch experiments were carried out in each cell: i) acetate as both electron donor and acceptor and ii) H_2 as sole electron donor and CO_2 as electron acceptor. CH_4 was analyzed after 24 hours in the four experiments. The cell containing the operating cathode produced a low amount of CH_4 with acetate. On contrast, higher CH_4 production was observed when H_2 and bicarbonate were the substrates, indicating that the most probable source of CH_4 were the hydrogenotrophic methanogens growing on the cathode. The cell with the operating anode did not produce CH_4, neither with H_2 nor with acetate, showing the probable absence of acetoclastic methanogens and hydrogenotrophic methanogens in the anode.

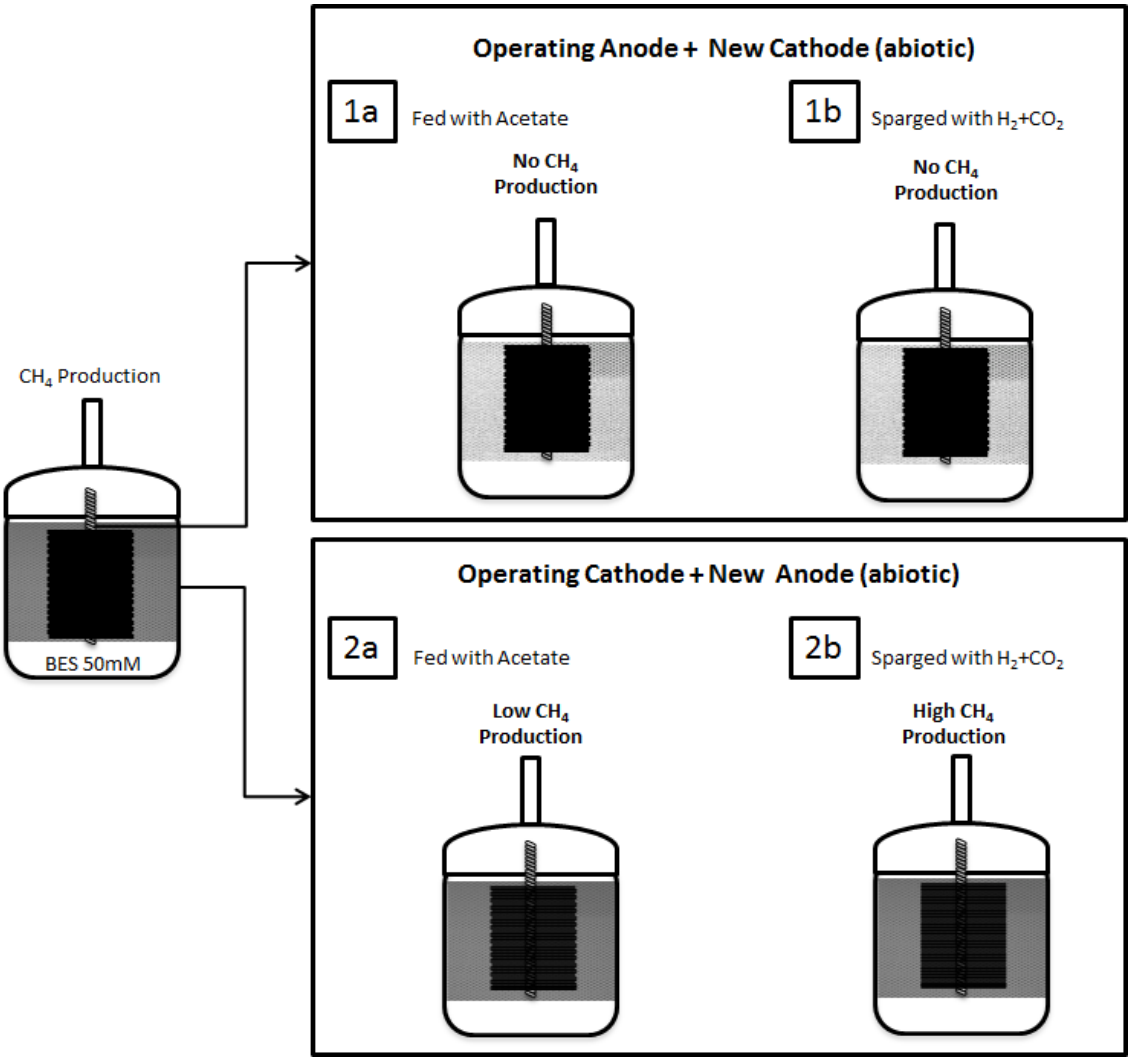

Figure 4.2 Qualitative experiment to demonstrate CH_4 production in the cathode. The operating anode and cathode were separated into two different cells with abiotic complementary electrodes fed with either acetate (Subfigures 1a and 2a) or H_2 and CO_2 (Subfigures 1b and 2b).

Characterization of the MEC microbial community through qPCR

In order to sustain the experimental observations explained before, microbial characterization of the biomass was carried out by qPCR (using the standard curves for qPCR obtained in section 3.1).

Figure 4.3 shows the qPCR results pointing *Geobacter*, *Methanobacteriales* and total *Archaea* in

the three DNA samples extracted from the MEC amended with 50 mM BES: anode, cathode and medium. *Geobacter* was predominantly present in the anodic biofilm (4.6 x 10^7 gene copies·mg^{-1}) compared with total *Archaea* and *Methanobacteriales* order (1.4 x 10^5 and 1.0 x 10^5 gene copies·mg^{-1}, respectively). On the other hand, the *Archaea* community was dominant in the cathodic biofilm (1.7 x 10^7 gene copies·mg^{-1}) with the hydrogenotrophic *Methanobacteriales* as the predominant order (1.5 x 10^7 gene copies·mg^{-1}), while the presence of *Geobacter* was much lower (3.5 x 10^5 gene copies·mg^{-1}). With respect to MEC medium, no significant differences were encountered among the microorganisms studied with a presence of approximately 5 x 10^5 gene copies·mg^{-1}.

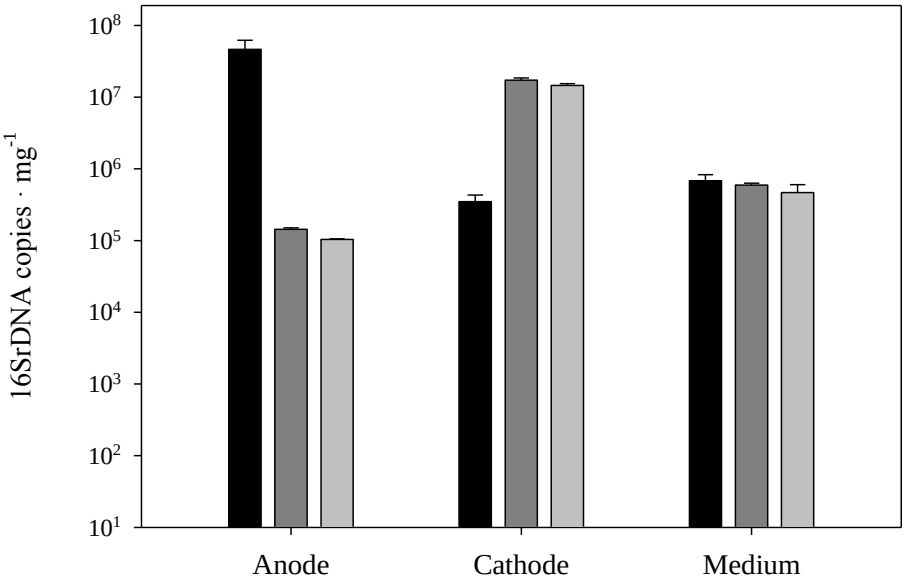

Figure 4.3. qPCR results for the cycle with 50 mM of BES concentration. qPCR distribution of *Geobacter* (black), total *Archaea* (dark grey) and *Methanobacteriales* (light grey) order on anode, cathode and medium in the MEC (mean of triplicate values ± standard deviation).

Pyrosequencing results

Anodic and cathodic DNA samples extracted from the MEC amended with 50 mM of BES were analyzed by high-throughput 16S rRNA gene pyrosequencing. Similar *Archaea* population was

found in both anodic and cathodic biofilms, most of them belonged to *Methanobrevibacter* genus (around 98% in both cases) (Figure 4.4). With respect to bacterial population, noteworthy differences were detected between anodic and cathodic biofilms. In this sense, the dominant population in the anode was the exoelectrogenic bacterium genus *Geobacter* (72 % of the total reads) (Figure 4.4). Other bacterium identified in this sample was *Proteiniphilum* (2 %). A 12 % of the population was designed as unclassified bacteria, since it was not possible to assign the sequences to any specific phyla. On the other hand, regarding the cathodic biofilm, the most represented bacterial genus was the homoacetogenic *Acetobacterium* (56 %). Almost 34% of the sequences could not be ascribed to any particular phyla. In both samples, a 1-2% of the sequences were included in the "others" category as its representation was below 1% of the total reads.

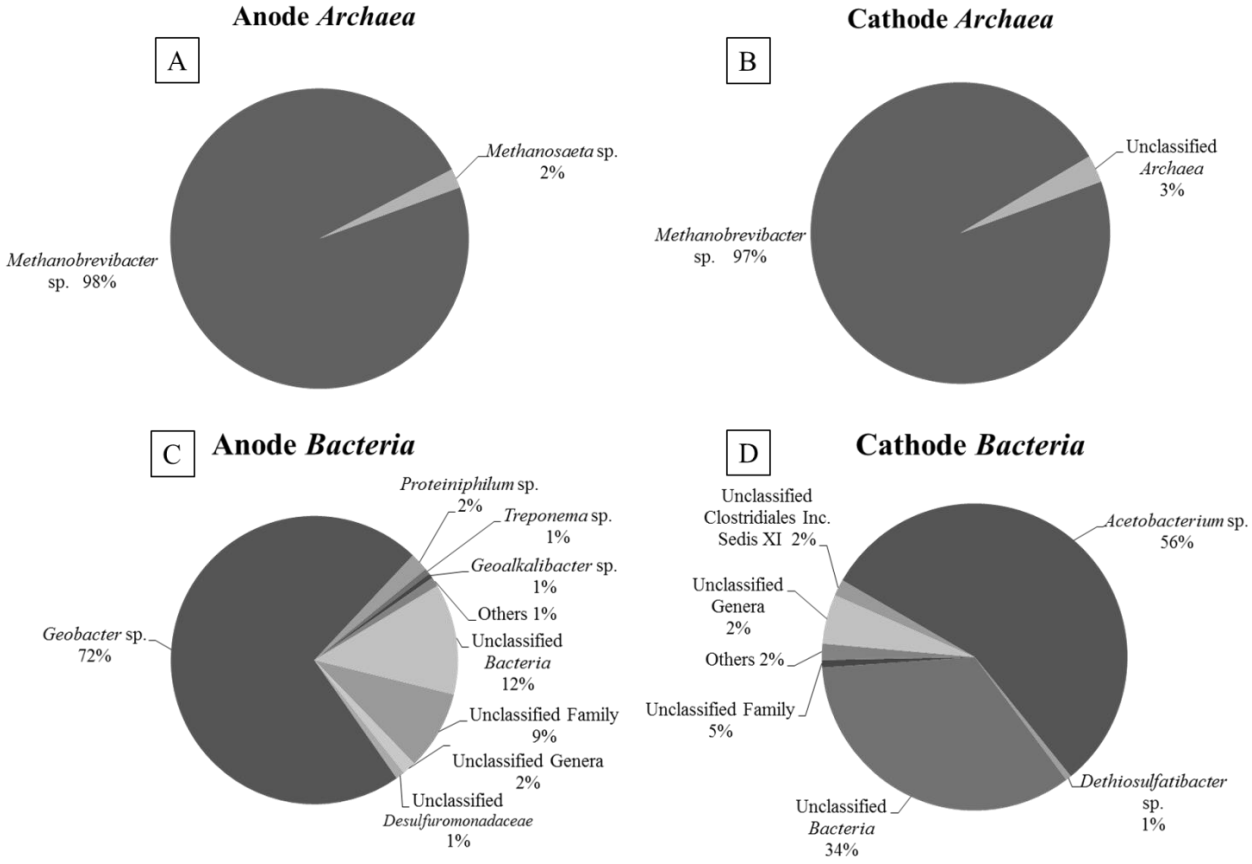

Figure 4.4. Pyrosequencing results of *Archaea* and bacterial communities. A, B: *Archaea* community. C, D: bacterial community. A, C: anodic biofilms. B, D: cathodic biofilms.

Scanning Electron Microscopy observations

Anode and cathode were sampled at the end of the period with 50 mM of BES and observed using SEM to characterize the biofilm structure and morphology (Figure 4.5). Graphite fibers of the anode were covered with an extracellular polymeric substance (EPS) while clear bacterial shapes of similar morphology were detected in the breaking points of the EPS structure. On the other hand, morphologically diverse bacteria (coccus and rods of different size) were observed on carbon cloth of the cathode, which showed a biofilm lacking in EPS with lesser biomass than the anode.

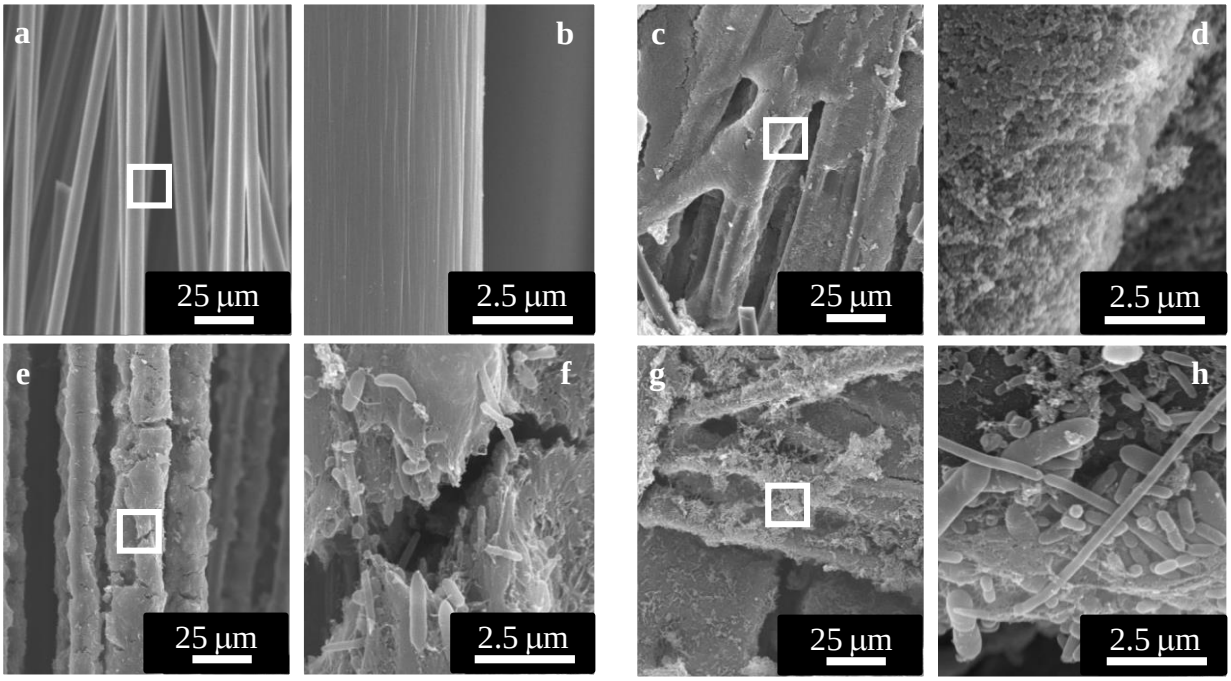

Figure 4.5. SEM images of anodes and cathodes. (a, b) abiotic anodic graphite fiber; (c, d) abiotic carbon cloth covered with carbon black and platinum; (e, f) anodic biofilm; (g, h) cathodic biofilm. White squares represent the zones that are magnified in images b, d, f, and h.

Methanogenesis at different BES concentrations

As methanogenesis detection at 50 mM of BES concentration was surprising, this process was monitored in additional experiments using higher BES concentrations (100 and 200 mM).

Current density and acetate concentration were monitored in each batch test (Figure 4.6). Cycle length increased from 160 h to 310 h when increasing BES concentration from 50 mM to 100 mM. When BES was further increased to 200 mM, the cycle length was extended and no decrease of current density was detected during the 450 h period that the cycle was operated. The decrease of current density observed for 50 mM and 100 mM experiments was linked to a decrease in acetate concentration. In spite of the current density observed for the experiment at 200 mM BES, very low decrease in acetate concentration was observed, as only 18 % of the initial acetate was consumed during the 450 h period. The fast increase in current density observed for example at time 120 h or 145 h in the 50 mM batch was due to the low acetate concentration linked to the manual agitation before medium sampling for acetate measurements.

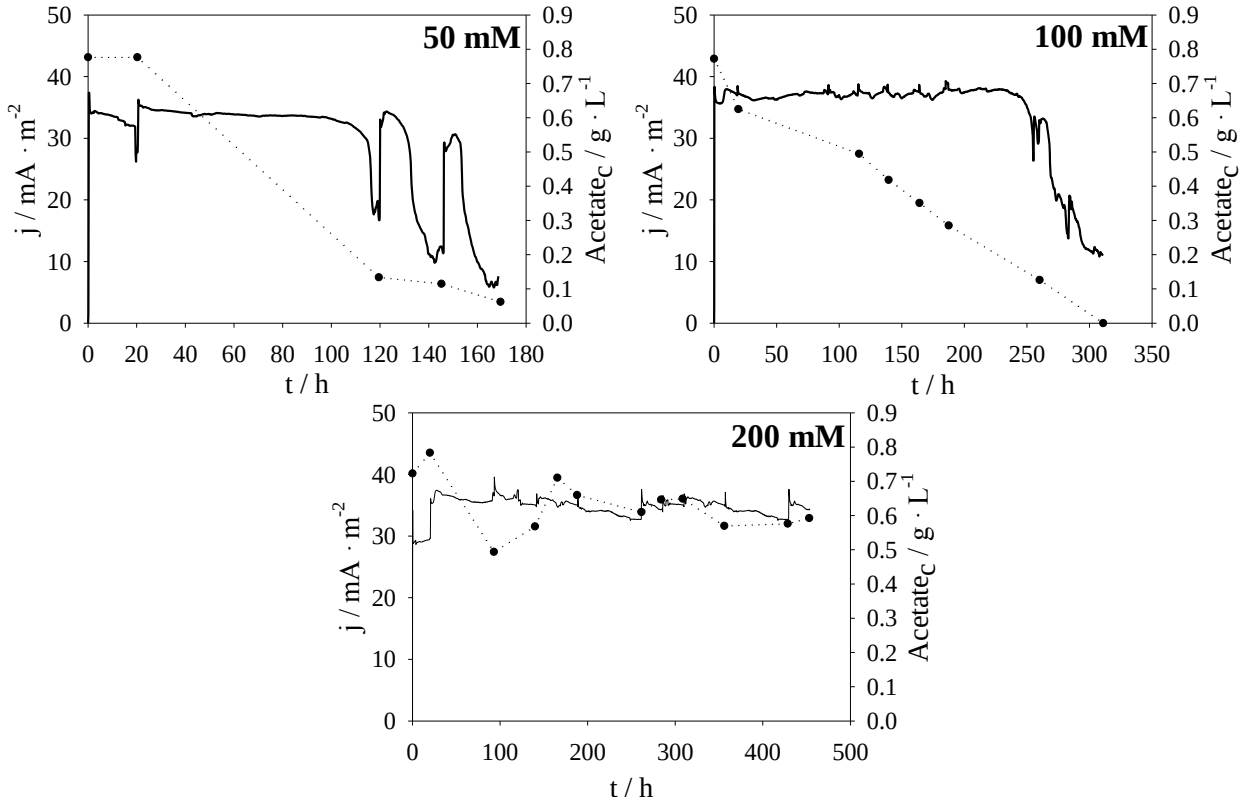

Figure 4.6. Current density (mA · m^{-2}) and acetate concentration (g · L^{-1}). Experimental current density (solid line) and acetate concentration (···●···) profiles from periods with different BES concentration: 50 mM, 100 mM and 200 mM.

Gas composition was monitored at each BES concentration (Figure 4.7). H_2 production was initially detected, reaching a peak in the first 20 hours of the cycle for each concentration step. During this first period, the rate of bioelectrochemical H_2 production was higher than that of hydrogenotrophic methanogenesis. A small amount of CH_4 was detected after the first 20 hours of batch-cycles, but it increased according to H_2 consumption, which was finally totally consumed and for this reason no H_2 was detected at the end of the cycles.

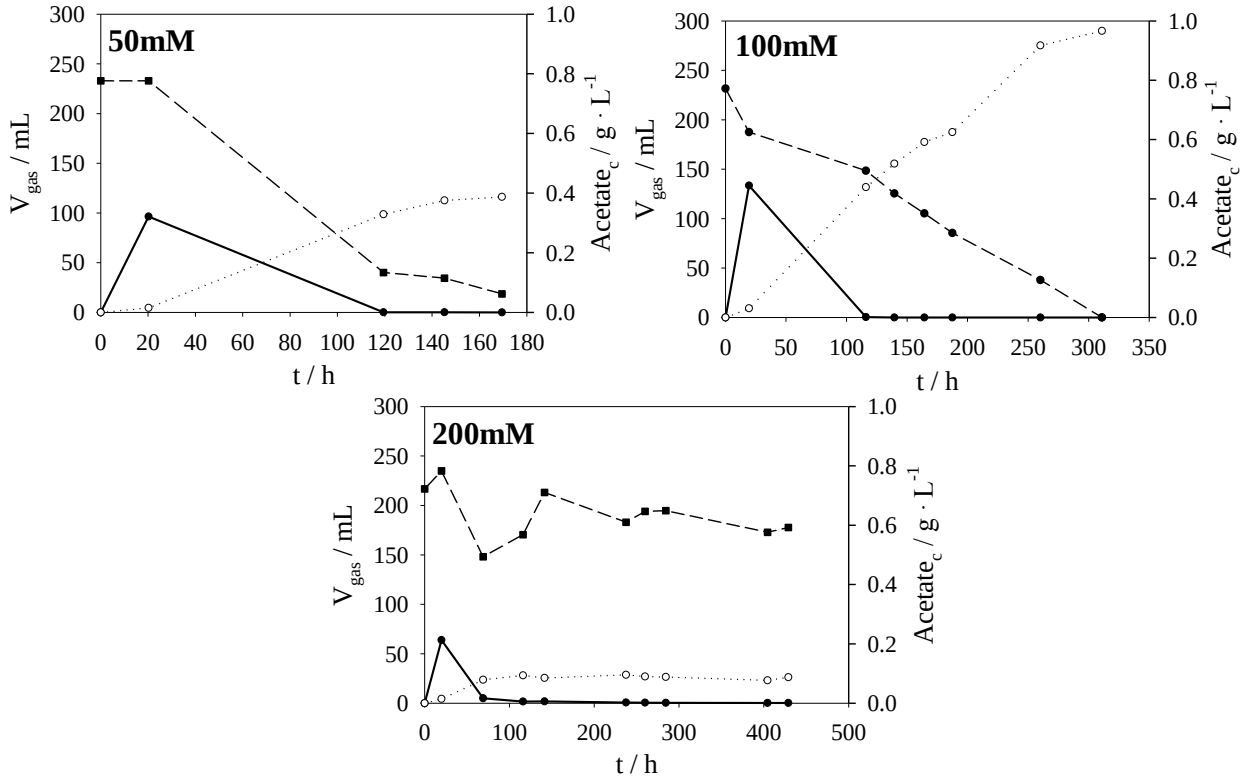

Figure 4.7. Gas composition. Experimental CH_4 -○- and H_2 -●- profiles from periods with different BES concentration: 50 mM, 100 mM and 200 mM.

Table 4.2 shows the CH_4 relative composition (Equation 4.1) over the time for the three cycles. With 50, 100 and 200 mM of BES concentration, the gas collected at the end of the cycles was mainly CH_4. The cycle with the initial concentration of 200 mM of BES was stopped after 450 h, but only a 23.5% decrease in acetate was observed (Figure 4.6). Homoacetogenic bacteria

recycled H_2 to produce acetate which was in turn consumed by exoelectrogenic bacteria, generating an extremely long cycle. Acetate consumption rate in this experiment was 6.4 $mg \cdot L^{-1} \cdot d^{-1}$ during the first 450 h. This would represent a cycle length of more than 112 days if this rate was extrapolated until complete acetate consumption.

Table 4.2. Methane relative composition (CH_4 / (CH_4 + H_2)). Gas composition in three cycles using different BES concentration was analyzed after 20, 120 and 160 h.

Time (h)	CH_4 / (CH_4 + H_2) (%)		
	50 mM BES	**100 mM BES**	**200 mM BES**
20	4.5	6.4	6.4
120	99.9	99.7	94.6
160	100	100	100

Table 4.3 summarizes the coulombic efficiency calculated for the three experimental periods. The values were higher than 100%, indicating the presence of H_2 recycling (further discussed below), which has been already reported for this type of systems [55]. Cathodic recovery (i.e. hydrogen recovered versus electrons arriving at the cathode) was almost null because the entire hydrogen production was being either recycled or used for methanogenesis.

Table 4.3. Experimental Coulombic Efficiency obtained in MEC using different BES concentration.

BES concentration	Coulombic Efficiency (%)
50 mM	194
100 mM	245
200 mM	1242

Finally, samples from the cathodic biofilm were collected at the end of each cycle with different concentration of BES and the presence of order *Methanobacteriales* was analyzed by qPCR. A slight decrease (less than 1 log) of this *Archaea* was observed at concentrations higher than 50 mM of BES but no differences were appreciable among 100 and 200 mM.

4.1.3. Discussion

This study reports CH_4 production in an MEC cell which was subjected to high BES concentration (50 mM) during a long-term period (4 months). Methanogenic activity under that BES concentration was surprising and, hence, it was investigated using two higher BES concentrations, 100 and 200 mM, for around 15 days each one. Figure 4.6 and 4.7 display the current density, acetate concentration and CH_4 and H_2 monitored for the whole cycles. To the best of our knowledge, this is the first MEC report with such high concentrations of BES. The high and increasing coulombic efficiency (being 194 % at 50 mM BES concentration and reaching 245% at 100 mM), suggested an important activity of homoacetogenic bacteria and H_2 recycling occurrence. In the cycle with 200 mM BES, methanogenesis was more inhibited as less methane was produced, but an important increase of homoacetogenic bacteria activity was observed, which is reflected in the extremely high CE of 1242% calculated for this experiment. Homoacetogenic activity was previously reported in membrane-less MEC [43,108], but our results demonstrate that the increase of BES concentration highly increments this issue: it results in a decrease of MEC performance because of H_2 recycling to acetate. This is corroborated with the increase of cycle length with 100 (310 h) and 200 mM BES (stopped at 450 h) (Figure 4.6). Despite this increase of homoacetogenic activity at high BES concentrations, qPCR showed a similar concentration of *Methanobacteriales*, perhaps, due to the inability of qPCR to discern DNA from active or inhibited microorganism. Longer operational periods with high BES concentration would be probably needed to observe a decrease in methanogens concentration.

The qualitative results shown in Figure 4.2 indicated that CH_4 production was most likely due to hydrogenotrophic methanogens attached to the cathode, in agreement with Sasaki et al. [109]. Both the qPCR and pyrosequencing results with 50 mM of BES concentration (Figures 4.3 and 4.4) confirmed this fact: the *Archaea* population of the cathodic biofilm was dominated by the

hydrogen-oxidizing genus *Methanobrevibacter*, of the *Methanobacteriales* order, which represented almost 98% of the total *Archaea*. The same values were detected in the *Archaea* of the anodic biofilm, where only 2% belonged to *Methanosaeta* (an acetoclastic methanogen). The lack of acetoclastic methanogens in single chamber MEC was already reported in Lee et al. [110] or Parameswaran et al. [43] even when BES was not added. They argued that acetoclastic methanogens have lower affinity for acetate when compared to acetate-oxidizing ARB (K_S = 177–427 vs 0.64 mg $COD \cdot L^{-1}$, respectively [111,112]), thereby enabling the anode as electron sink rather than methanogenesis in acetate limited environments. The MEC operation in batch mode with 252 mg $COD \cdot L^{-1}$ of initial acetate led to substantial acetate limitation in most of the reaction period and hence, the competition for acetate between ARB and methanogens seemed to be the key for the acetoclastic washout rather than different BES inhibition effect on H_2 - vs. acetate-oxidizing methanogens.

However, qPCR results (Figure 4.3) show that the presence of *Archaea* in the anode was two orders of magnitude lower than in the cathode, and hence its activity in the anode was negligible when compared to the abundance of *Geobacter*, the most common exoelectrogenic microorganism found in acetate-fed bioelectrochemical systems [93]. The genera of *Geobacteraceae* family, like *Geobacter*, are able to oxidize acetate, colonize the electrode surface and conserve energy to support growth from electron transport to the electrode, using it as the sole electron acceptor. This gives them a competitive advantage over other microorganism in bioelectrochemical systems as MFCs and MECs [18].

On the other hand, the dominant bacterial genera identified in the cathodic biofilm was *Acetobacterium* (a homoacetogenic genus), able to oxidize H_2 and reducing CO_2 to produce acetic acid [106]. However, according to the presented pyrosequencing results, the ratio of *Methanobrevibacter* sp. with respect to *Acetobacterium* sp. in the cathode is ca 5:1. *Acetobacterium* was already isolated in MECs systems in previous studies [55,108]. Their presence in the cathodic biofilm, in addition to hydrogenotrophic methanogens, can be explained by the continuous H_2 production in the cathode and because of its high concentration in the medium during the first hours of the cycle.

Once methanogens were detected, their presence in a cell with such high BES concentrations should be examined. Three different hypotheses could be put forward: i) biofilm mass transfer resistance; ii) BES degradation, and iii) microbial adaptation to high BES concentrations [113].

One hypothesis could be the mass transfer resistance of the EPS matrix of the biofilm. When this matrix is very thick, among other functions, it can hinder access and diffusion of chemical compounds (like antimicrobial agents) to the bacterial cells [114] leading to partial BES penetration and, hence, producing biofilm stratification with methanogens in the inner side. This would explain common BES observations: i) inhibition being effective in immature biofilms (i.e. short-term exposure), and ii) difficulty in reducing methanogens concentration when they are dominant in the system. Nevertheless, thanks to the SEM images, this hypothesis seems improbable since the cathodic biofilm looks very thin and apparently lacking of EPS.

Another hypothesis could be that the long-term exposure to BES resulted in the growth of microorganisms capable of BES degradation [113]. According to the pyrosequencing results, this hypothesis also seems improbable since any microorganism capable to either reduce or oxidize the sulfonate group of BES was detected in a high proportion. Moreover, this hypotesis serves as an input for the study presented in section 4.2.

The last hypothesis could be that the long-term exposure of the biofilm to BES led to the development of some resistance to BES. This resistance has already been reported [115–117] and would be based on an inability to transport BES into the cell. As a structural analog of CoM, BES is a specific inhibitor of the terminal step in CH_4 biosynthesis and some methanogens are non-CoM dependent because they can synthesize CoM [118] and as such they can develop BES resistance [113]. Accordingly, development of BES adaptation could explain the experimental results obtained. The causes of this potential adaptation deserve further studies, but the operational conditions of our system (i.e. long cycles, high BES concentration and high cathodic hydrogen retention time) could be plausible reasons behind these results.

4.2. BES degradation in bioelectrochemical systems

The content of this section was partially published in Bioelectrochemistry as: L. Rago, J. Guerrero, J. A. Baeza, A. Guisasola (2015), 2-Bromoethanesulfonate degradation in bioelectrochemical systems. Bioelectrochemistry, 105, 44–49. [48]

4.2.1. Specific experimental procedures for section 4.2

MFC and MEC operated in batch mode were used in this study. Two c-MFCs (MFC-A and MFC-B, see Chapter 2 for further details) were inoculated with mixed liquor from a i-MFC. c-MFCs were run for more than 40 days (8-10 batch cycles) and, then, converted into c-MEC (MEC-A and MEC-B) by avoiding oxygen presence in the cathode with a methacrylate cover and applying a constant potential of 0.8V. Initial and final BES concentration measurements in MFC cycles started after a stabilization period of 4 cycles. After the conversion from MFC to MEC, BES concentrations were monitored from the first cycle onwards.

Some MEC experiments were conducted on a 1.3L MEC (the concentric MEC detailed in section 4.1) to avoid limitations of volume of sample withdrawal and to obtain experimental BES profiles in time (Figure 4.9).

Acetate was used as the sole carbon source with an initial concentration around 20mM. Initial BES concentration in each cell was 10, 50 or 100 mM depending on the specific experiment. The medium used was the 100 mM phosphate buffer prepared described in chapter 2. The batch cycle duration was between 3 and 5 days.

Two different controls were designed. MFC-B was used to evaluate BES degradation under starvation conditions, using the same media but without substrate addition. The second control was an abiotic-MFC that was operated under the same MFC conditions to appraise chemical BES oxidation.

Ion chromatography

The samples for BES and Br^- analyses were collected from the medium at different steps of the bioelectrochemical cycles. Then, samples were serially diluted, filtered (0.22 μm) and analyzed using Ion Chromatography, a Dionex ICS-2000 (RFIC) with an Ultimate 3000 Autosampler Column Compartment, an IonPac AS18 column and an IonPac AG18 pre-column (ThermoScientific, USA). BES and Br^- concentration were quantified by using two standard curves that ranged from 10 μM to 50 μM using sodium-BES or potassium bromide. The R^2 obtained for BES and Br^- were 0.9974 (n=5) and 0.9996 (n=5).

DNA extraction

The cathodic biofilm of MFC-A was collected in a sterile Eppendorf from the carbon cloth with a sterile spatula, centrifuged at 10000 g (Thermo Scientific Hareus Pico17, USA) and the supernatant was eliminated to remove residues from the growth medium. DNA was extracted

using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) as described in section 4.1.

High-throughput 16S rRNA gene pyrosequencing

Pyrosequencing was performed in a 454 Titanium FLX system by the Research and Testing Laboratory (RTL, Lubbock, TX) and the results were analyzed following the protocol previously described in section 4.1. However, DNA samples were analyzed by using a different couple of primers of the bacterial 16S rRNA gene:

- 357F-926R which comprising the V3-V5 regions of the bacterial 16S rRNA gene [119];

4.2.2. Results and discussion

Potential BES degradation was studied in the bioelectrochemical systems (MFCs and MECs). Initial and final BES concentrations were measured in several cycles from MFCs and MECs using different cells (MFC-A, MFC-B, MEC-A, MEC-B and the concentric MEC). The amount of BES degradation in these cycles, with an initial BES concentration of 50 mM, is shown in Figure 4.8. Significant BES degradation was observed in MFCs. Conversely, variation in BES level was negligible in the MECs. An abiotic-MFC was monitored to discard any possibility of chemical or electrochemical degradation of BES under the experimental conditions (Figure 4.8). BES concentration did not decrease in this cell, indicating that the bacterial community was responsible for the BES degradation under the MFC-mode.

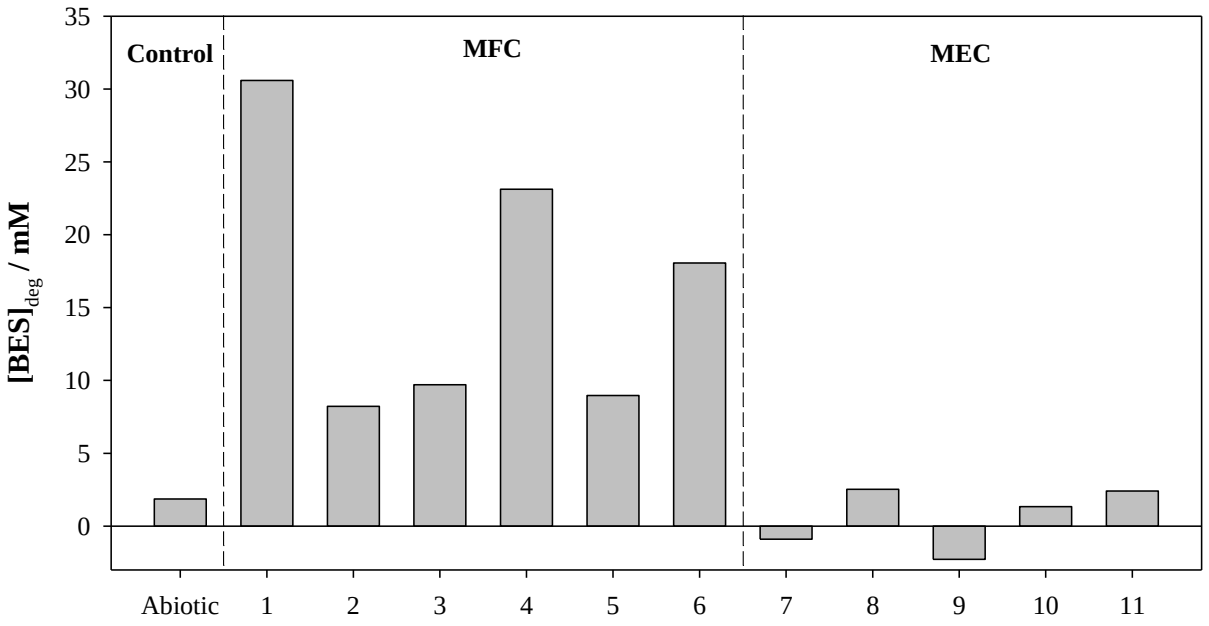

Figure 4.8. Experimental BES degradation in different MFC and MEC cycles for an initial BES concentration of 50mM. BES concentration was measured at the beginning and end of each batch cycle (3–5 days each one). The first vertical bar represents the abiotic-MFC control. The experiments were performed in different cells: 1 (MFC-A), 2–6 (MFC-B), 7–8 (MEC-A), 9 (MEC-B), 10–11 (the concentric MEC).

BES and Br^- were measured along some batch cycles in order to gain insight into the process. Examples of the experimental profiles obtained from MFC-A, MFC-B and the concentric MEC are shown in Figure 4.9. Simultaneous Br^- formation was linked to BES degradation (at a rate between 2 and 6 $mM \cdot d^{-1}$) and was only observed in MFCs. The average Br- recovery (i.e., molar ratio of Br- released to BES degraded) in all MFC experiments was 67 ± 16%. For the particular cases displayed in Figure 4.8, Br^- recovery was 70.5 (MFC-10 mM), 86.8 (MFC-50 mM) and 64.7% (MFC-100mM). Br^- formation suggests cleavage of the $–CH_2–Br$ chemical bond. The presence of other intermediate compounds resulting from BES degradation may be responsible for the imbalance of Br^- recovery.

BES was also monitored under starvation conditions (i.e., without substrate addition) and in MFC-B cell with an initial BES concentration of 100 mM (Figure 4.9). Almost 30% of this BES was degraded in 144 h with a BES degradation rate that was close to that under feast conditions. This indicated that BES degradation was independent of the anodic activity and acetic acid presence. Nevertheless, because no change in BES concentration was observed in the abiotic-MFC, the exact degradation route might be related (entirely or partially) to a biologically catalyzed reaction.

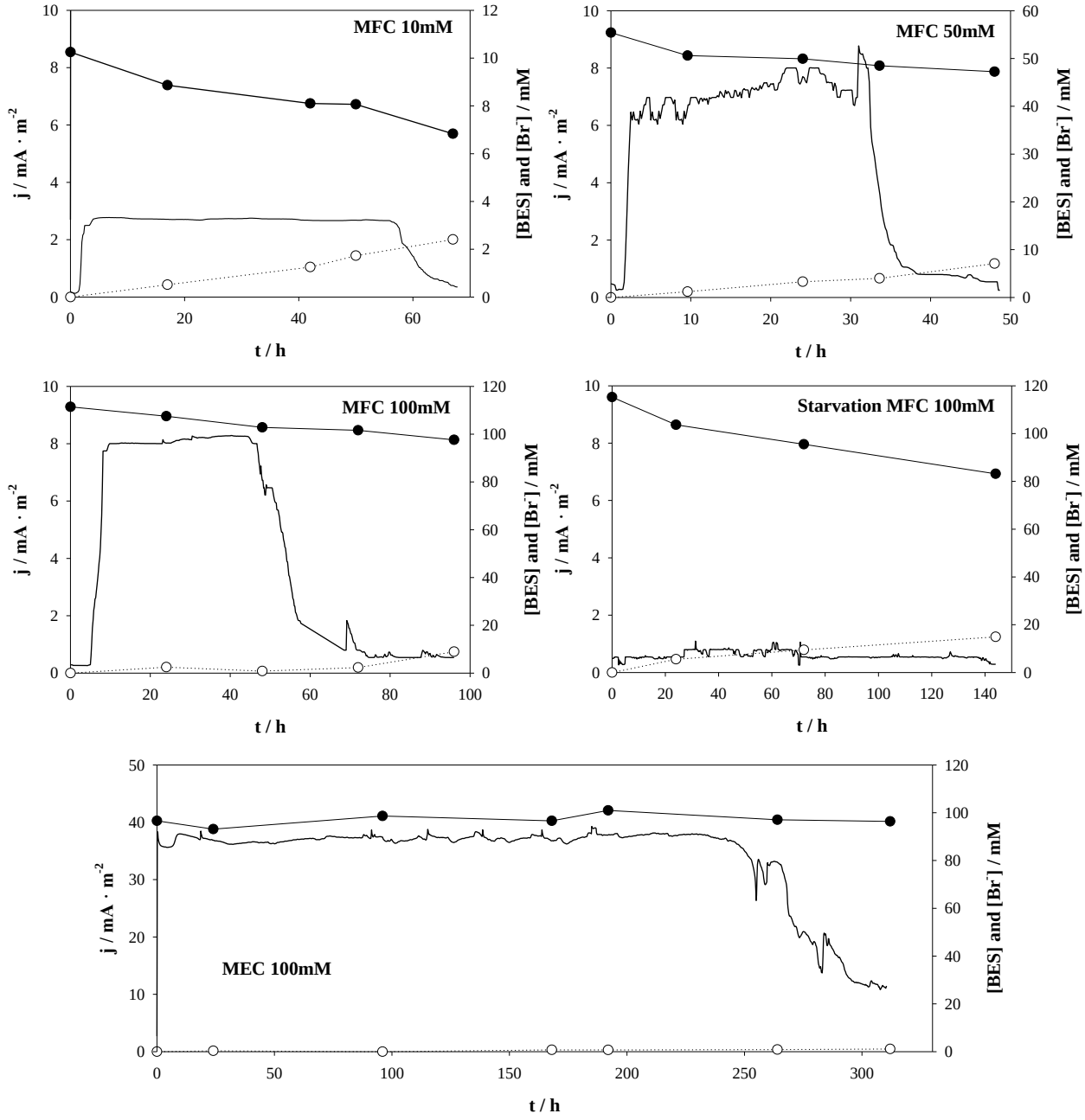

Figure 4.9. Cycle profiles in MFC and MEC. Experimental profiles of 2-bromoethanesulfonate (BES;●), Br^- (○) and current density (solid line) for experiments monitored in different cells: MFC 10 mM was conducted in MFC-A; MFC 50 mM, 100 mM and starvation (100 mM BES and without substrate addition) in MFC-B; MEC 100 mM in the concentric MEC.

The fact that BES degradation was only observed in MFCs was surprising because the MECs used here consisted of the same anode, medium and cathode as the BES-degrading MFCs, except for the fact that the cathode was not open to the air and there was an applied potential (0.8V) to drive hydrogen production. Hence, the entire microbial community, part of which was able to degrade BES, was present in both the MFCs and MECs. Thus, it is likely that the BES degradation we observed was due to the MFC cathodic conditions. Air-cathodes are known to allow some oxygen diffusion to the cell, resulting in a cathodic biofilm formation. On a mid-term basis, the cathodic biofilm acts as an oxygen barrier that prevents oxygen from entering the anodic chamber [120,121]. Indeed, when the biofilm from an air-cathode is manually suppressed, oxygen penetrates through the cathode to the rest of the cell and subsequently decreases cell performance [120]. Cathodic biofilms present a multilayer structure according to dissolved oxygen penetration, which enables the presence of a wide range of genera (e.g., strictly aerobic, microaerophilic, aerotolerant and strictly anaerobic).

The microbial composition of the cathode from an MFC was also analyzed to identify the potential microorganisms and mechanisms involved in BES degradation. Figure 4.10 shows the pyrosequencing results obtained in MFC-A, which had been working with 10 mM BES media for 40 days. There was no dominant population in the cathodic biofilm. *Azoarcus* sp. was the most significant, which was responsible for only 10% of the sequences found. *Azoarcus* sp. is a genus of nitrogen-fixing bacteria common in soils and contaminated waters, usually with a strictly aerobic metabolism, exhibiting microaerophilic growth and growing well on salts of organic acids, but not on carbohydrates [122]. *Fusibacter* sp. (6%) is a strictly anaerobic member of the *Clostridium* phylum. The bacteria of this genus can use thiosulfate and sulfur as electron acceptors. *Pseudomonas* (4%) is a well-defined, generally strict aerobic genus [123]. *Proteiniphilum* (4 %) was another identified strictly anaerobic bacterium [124]. *Desulfovibrio* (4%) is the most thoroughly studied genus among sulfate-reducing bacteria (SRB) and very common in environmental samples [125]. Bacteria of this genus are generally considered strictly anaerobic, but there are exceptions demonstrating that some species of this genus could be aerotolerant at the expense of having limited growth [126]. *Desulfomicrobium* (2%) is another strictly anaerobic genus of SRB. Five percent of sequences were of the *Alcaligenaceae* family (unclassified genera). Finally, genera making up less than 1% of total composition were classified as “others” (12% of total sequences)

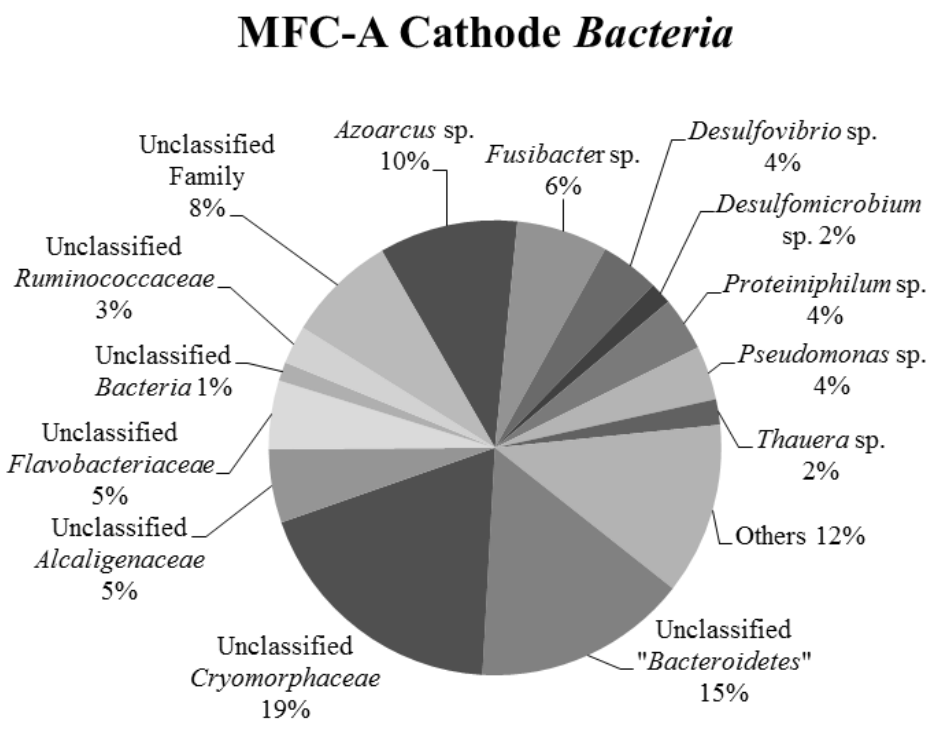

Figure 4.10. MFC microbial community. Cathodic genus microbial distribution in MFC-A through high-throughput 16S rRNA gene pyrosequencing. Genera which constituted less than 1% of total sequences were classified as others.

Taken together, our data indicate that BES is only degraded when the biofilm had oxic conditions. Sulfonates can be used as a carbon and energy source or used for sulfur scavenging under sulfate-limiting conditions. While desulfonating enzymes are regulated by induction into aerobic bacteria, it is unclear whether this sulfur scavenging ability is found when no oxygen is present [127]. Kertesz [128] showed that *Pseudomonas aeruginosa* (present in Figure 4.10) can use a wide range of alkanesulfonic acids as a sulfur source as part of the sulfate-starvation-induced stimulon. Uria-Nickelsen et al. [129] showed how a variety of sulfonates with a similar structure to BES (e.g., taurine) could be used as a sulfur source under oxic conditions by selected enteric bacteria, but not under anoxic conditions. Finally, Thurnheer et al. [130] showed that a strain of *Alcaligenes sp*. required oxygen presence in the desulfonation process in order to cleavage the C-S bond. This oxygen requirement could explain the non-observed BES degradation under MEC conditions.

While BES degradation may not be significant in short-term experiments, long-term BES dosage would favor the development of a sulfonate-degrading community; modifying the biomass distribution and resulting in a lower BES concentration than desired in long-term experiments. This decrease may lead to unexpected methane production.

4.3. Conclusions

Despite the high concentration of BES used in a single chamber MEC, methanogenesis was not totally inhibited. The reason of this was likely due to the capacity of some *Archaea* (hydrogen-oxidizing genus *Methanobrevibacter*, of the *Methanobacteriales* order) to resist high BES concentration up to 200 mM.

These results demonstrate that H_2 production in single chamber MEC is seriously challenged by CH_4 production even when operating at 100 mM BES concentration. Moreover, at higher BES concentration (200 mM), methanogenesis activity decreased resulting in an increase of H_2 recycling by homoacetogenesis. High presence of homoacetogenic bacteria undermines the efficiency of MEC to H_2 production. Further studies will be also needed to prevent homoacetogenic bacteria from undermining methane production in MECs designed to favor hydrogenotrophic methanogenesis.

BES degradation was suggested, in section 4.1, as one of the hypotesis to explain BES resistance. However, the study presented in section 4.2 demonstrated that BES degradation was only observed under an air-cathode MFC configuration. Thus, strict anaerobic systems, such as an MEC, would not be challenged by the described phenomenon.

The demonstration of BES degradation in bioelectrochemical systems, reported in section 4.2 has several practical implications. BES degradation is a biologic process and, thus, requires the growth of a BES-degrading community. Periodic BES dosage in long-term batch experiments would be required to avoid methanogenesis, but this could lead to an increased fraction of BES-degrading microorganisms.

CHAPTER 5

Pure and mixed alkaline cultures

MXCs: development and study

Motivation

The bacteria from genera of *Geobacteraceae* and *Shewanaellaceae* families are the most studied ARB [25]. *Geobacter* genus is commonly found in acetate-fed high current intensity producing MXCs. Nevertheless, several different ARB can be found in MXCs depending on the substrate [45], the inoculum [29] and experimental conditions [29,50].

Most of these studies are based on moderate pH conditions. However, bioelectrochemical systems should extend the range of their practical capabilities to include wastewater treatment and energy generation under different pH scenarios. Given the results reported so far, understanding exoelectrogenic activity under alkaline conditions is very stimulating since: i) Badalamenti et al. [24] found the highest current densities ever achieved by pure cultures under alkaline conditions with a pure culture of the genus *Geoalkalibacter*; ii) *Geoalkalibacter* are also very attractive since they are halophilic and give successful results under high-saline conditions [24,29,131,132]; iii) alkaliphilic environments may also be favorable in order to prevent acidity buildup in MXCs [133,134]; iv) an alkaline environment could create a more selective and favorable environment in the competition between methanogens and exoelectrogens for the electron donor; and v) alkaline bioelectrochemical systems can be a suitable technology for the direct treatment of alkaline wastes (e.g. beamhouse wastewaters from leather tannery or wastewaters with glycerol produced in alkaline biodiesel production).

Thus, the aim of this chapter was to compare the long-term performance of bioelectrochemical systems inoculated with pure (section 5.1) and mixed cultures (section 5.2), under alkaline conditions. The results obtained in section 5.2 were also compared to a conventional reactor operated at neutral pH.

5.1. Opportunities of *Geoalkalibacter Ferrihydriticus* for bioelectrochemical hydrogen production

The study presented in section 5.1 was carried out in the Swette Center for Environmental Biotechnology in Arizona State University under the supervision of Dr. César I. Torres and with the help of Dr. Sudeep C. Popat and Rachel Yoho.

5.1.1. Specific experimental procedures for section 5.1

Media and pure culture conditions

Geoalkalibacter ferrihydriticus DSM 17813_T inoculum was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The pure culture tubes were maintained in anoxic condition in butyl rubberstoppered Hungate tubes using 1 ml of inoculum in 10ml of total volume of *Glk. ferrihydriticus* medium with a final pH 9.3. The medium was prepared like previously described in [24]. The anoxic medium contained (per liter of deionized water): 0.2 g KCl, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.2g KH_2PO_4, 1.0g NaCl, 1.36g sodium acetate trihydrate, 1ml of trace elements solution and 0.5ml of selenite-tungstate solution (Widdel and Bak, 1992). After being autoclaved, several anoxic stock solutions were added per liter of medium: 20 ml of Na_2CO_3 150g/l, 111ml of $NaHCO_3$ 90 g/L, 5 ml Wolfe's vitamin solution (ATCC, Manassas, VA) and 10 ml of NH_4Cl 50g/l. Finally the media was bubbled with 80%N_2-20%CO_2 mixed gas. 50 mM Fe(III) oxide as the electron acceptor from a sterile anoxic stock solution prepared as previously described in (Lovley and Phillips, 1986) were added directly in the pure culture tubes. Incubation temperature was maintained constant at 30ºC in all experiments. Same medium (without the use of Fe(III) oxide solution) and same temperature were used for MEC experiments.

Pure culture MEC construction and inoculation

Three 100 ml modified lab bottles were used to build three single-chambers pure culture MECs: Gold-A, Gold-B and Gold-C. A gold fill circle (3.14 mm^2 area) was used like anode and a thin nickel (~ 8 mm long) wire was used like cathode. An Ag/AgCl reference electrode (BASi, West Lafayette, IN) was dipped a few minutes in 70% (vol/vol) ethanol, flicked dry, and inserted into the autoclaved reactors.

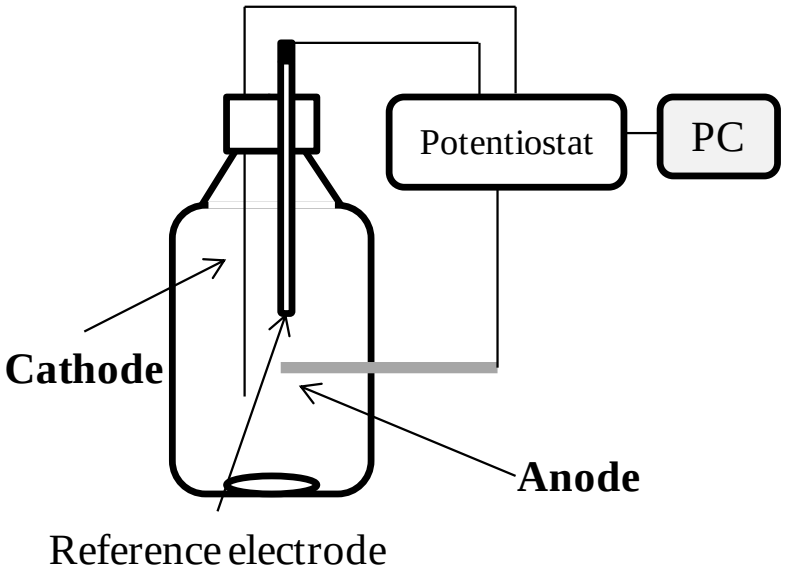

Figure 5.1. Schematic representation of pure culture MEC

The reactors were filled in a biosafety cabinet by flowing pressurized media through sterile Norprene tubing (Cole Parmer, Vernon Hills, IL) and then, they were briefly bubbled with N_2 using 0.22-µm-pore-size filters. The reactors were inoculated using 2 ml from *Glk. ferrihydriticus* (DSM 17813) pure culture tubes. Magnetic stirring at 150 rpm was used for agitation.

Chronoamperometry and cyclic voltammetry.

All experiments were performed using a VMP3 digital potentiostat (Bio-Logic USA, Knoxville, TN) and using an Ag/AgCl aqueous reference electrode (BASi, RE-5B, 3M KCl filling solution, +270mV vs SHE [135]). Anodes in Gold-A and Gold-B were poised at -0.13 V versus SHE and Gold-C at 0.07 V versus SHE.

Cyclic voltammograms (CVs) were collected for each reactor at nine different times of the biofilm growth. For each growth-point, two CV replicates were recorded from the anode open circuit potential (around -0.38 V versus SHE) to 0.07 V versus SHE (-0.13 V versus SHE for the first five growth-points CVs of Gold-C, to preserve the immature biofilm). Nevertheless, the two CVs replicates were almost identical and for this reason only the second duplicate was presented in this work.

In all graphs plotted in this study, the potentials were converted from Ag/AgCl to SHE.

pH experiments

pH experiments were conducted on Gold-A. NaOH or HCl 10M (0.3-0.5 ml) anoxic stock solutions were added to adjust the pH to the desired value under anoxic conditions. The pH was measured with a microelectrode pH probe (Cole Parmer) and a digital pH meter (Thermo Scientific).

5.1.2. Results and discussion

Development of exoelectrogenic activity

Anodes in Gold-A and Gold-B were poised at -0.13 V (vs. SHE) under alkaline condition (pH 9.3) after the inoculation. Exoelectrogenic activity was detected with current density production approximately six days after the inoculation in Gold-A, and after eight days in Gold-B (Figure 5.1). This shift was detected in spite of using theoretically the same experimental conditions

probably due to a highest content of *Glk. ferrihydriticus* in the inoculum obtained from pure culture tubes.. The highest current density value was obtained after eight days in Gold-A (around 10 $A \cdot m^{-2}$) and in 12 days in Gold-B (around 8 $A \cdot m^{-2}$).

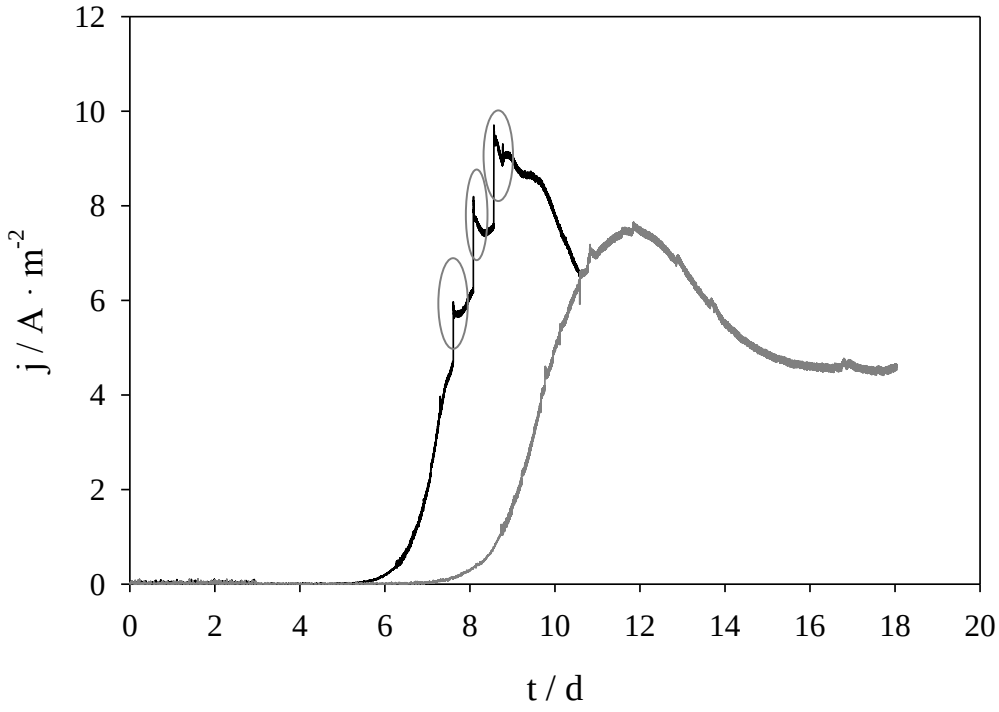

Figure 5.1. Chronoamperometry obtained from *Glk. ferrihydriticus* Gold-A (black line) and Gold-B (grey line). Both anodes were poised at -0.13 V versus SHE, following inoculation with cells grown on Fe(III) oxide. The circles indicate current density jumps obtained after cyclic voltammograms.

After reaching the highest values, both cells showed a decreasing trend of the current density, which in the case of Gold-B stabilized at less than 5 $A \cdot m^{-2}$, a 60% of the maximum value (8 $A \cdot m^{-2}$) This peculiar behavior of the biofilm, in which the maximum current density is higher than the current density of the saturated biofilm, was previously reported in *Geoalkalibacter* species [24,131]. These results suggest that the maximum current density production

corresponded to an optimum biofilm thickness and, thus, we hypothesized that the biofilm may be mass transport-limited at steady state operation.

A third MEC, Gold-C, was built and inoculated and the anode was poised at 0.07 V (vs. SHE). Current density production was achieved quickly after less than two days as shown in Figure 5.2. Gold-C shows the faster and more stable performance, if compared with the other two MECs, Gold-A and B. The highest current density was obtained after 8 days, and it reaches a stable value (around 8 $A \cdot m^{-2}$) suggesting that the applied potential used for this MEC was better for the *Glk. ferrihydriticus* growth in these conditions. That could be due to the specific electron transport mechanism used by these bacteria. Further studies are needed to better understand this result, using for example other anode potentials.

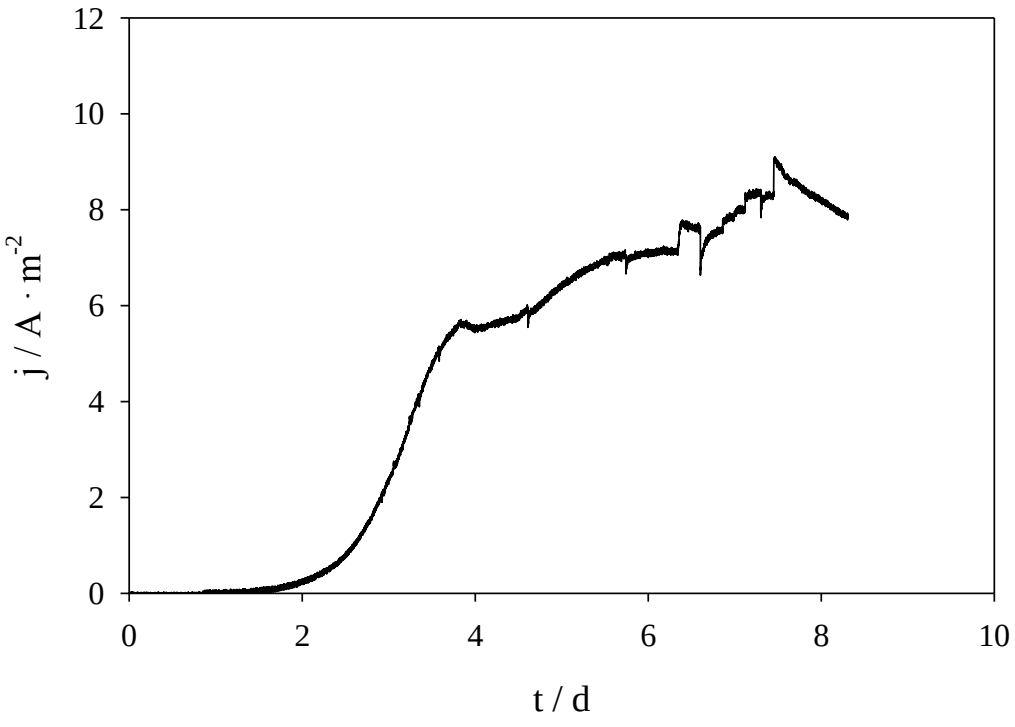

Figure 5.2. Chronoamperometry obtained from Gold-C, operated with an anode applied potential of 0.07 V (vs. SHE).

Cyclic voltammograms at different times of the biofilm growth

Different cycling voltammograms (CVs) were recorded on the three cells in at different points of biofilm growth. In Gold-A, the first CV were obtained when the cell was producing 0.4 $A \cdot m^{-2}$ (after 6 days) of current density and the last one was obtained when the cell was producing 7.5 $A \cdot m^{-2}$ (after around 8 days; Figure 5.3).

The CVs showed unique form which presented a reverse curve higher than the forward one, which indicated that the *Glk. ferrihydriticus* biofilm was growing faster when the anode potential was higher. Observing Gold-A growth curve (Figure 5.1), the current density showed peculiar current density jumps (grey circles). These jumps happened after recording the CVs from the three last growth-points (when the cell was producing 4.7, 6.2 and 7.5 $A \cdot m^{-2}$ of current density) and they were due to the faster biofilm growth during the CVs (around 10 minutes of duration). The highest current density reached in the last CV was around 10 $A \cdot m^{-2}$ for Gold-A (Figure 5.3). These results were really interesting because they confirmed that an anode applied potential of -0.13 V (vs. SHE) was not the optimal potential for the *Glk. ferrihydriticus* pure culture reactor.

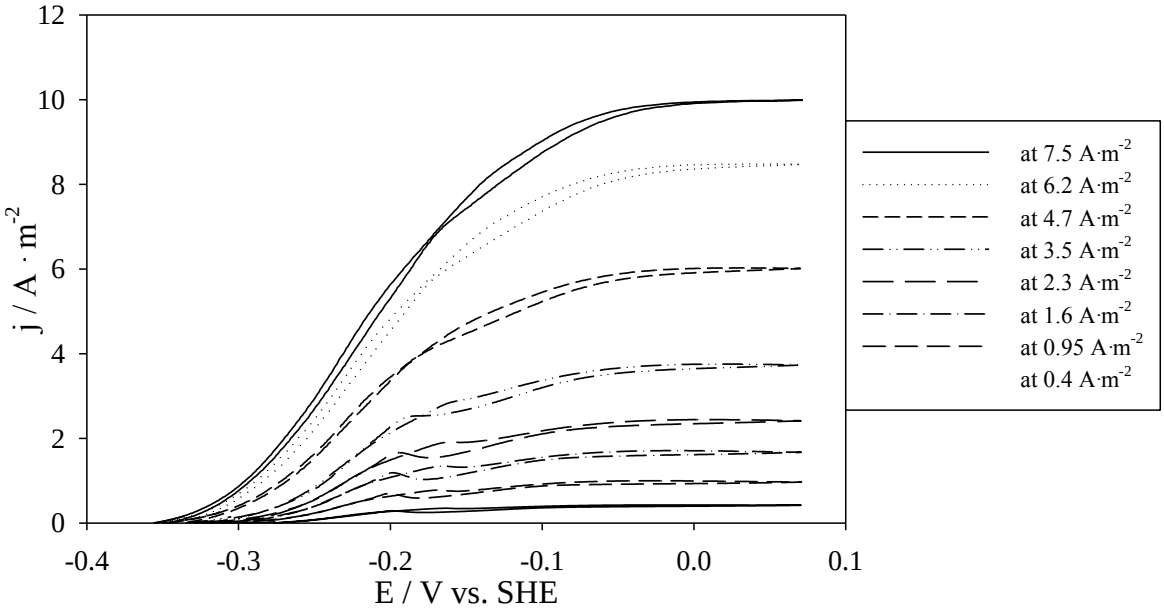

Figure 5.3. CVs monitored for Gold-A reactor at eight different biofilm growth-points from the anode open circuit potential (around -0.38 V versus SHE) to 0.07 V versus SHE.

The chronoamperometry results for Gold-B cell are shown in Figure 5.4. The anodic growth was slower than Gold-A during the inoculation period in spite of the same applied anodic potential. Gold-B anodic biofilm growth was more regular than Gold-A and, thus, those peculiar jumps previously discussed were not present. Gold-B and Gold-A after reaching the highest current density showed the same decrease of current density.

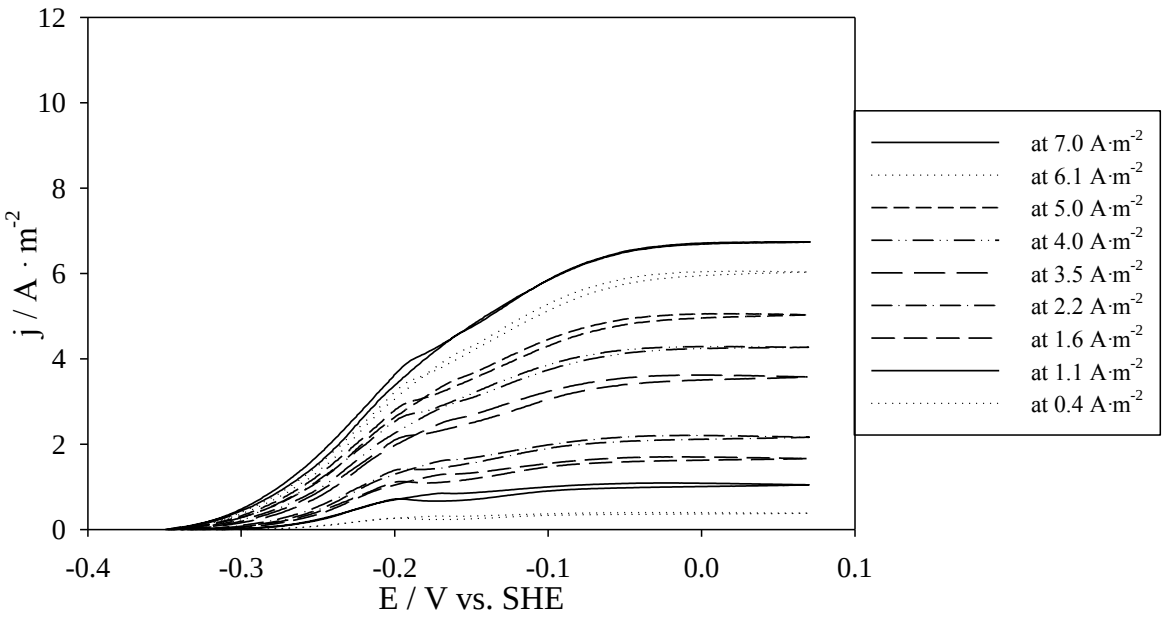

Figure 5.4. . Cyclic voltammograms (CVs) monitored for Gold-B reactor at nine different biofilm growth-points from the anode open circuit potential to 0.07 V (vs. SHE).

As explained before, Gold-C anode was poised at 0.07 V (vs. SHE) to confirm what was observed during the inoculation with Gold-A and Gold-B. For the first five growth-points, two CVs replicates (Figure 5.5, only the second replicate was shown) were recorded from the anode open circuit potential (around -0.38 V versus SHE) to -0.13V versus SHE to preserve the immature biofilm. The curves obtained from Gold C showed a totally different shape than those from Gold-A and Gold-B (*e.g.* forward curves obtained were not highest than reverse curves). After this first biofilm growth period, CVs were performed between anode open circuit potential

to 0.07 V (vs. SHE). In all cases forward curves reached higher current density values than reverse curves. That suggested that the biofilm was not growing faster during the CV analysis, in contrast to what was possible to observe in Gold-A experiments. These results indicated that the anode applied potential used for Gold-C was more adequate for the faster and high performance inoculation of *Glk. ferrihydriticus* pure culture MECs. The highest current density obtained during the last CVs was around 9 $A \cdot m^{-2}$, according to the maximum current density obtained during chronoamperometry analysis (Figure 5.2).

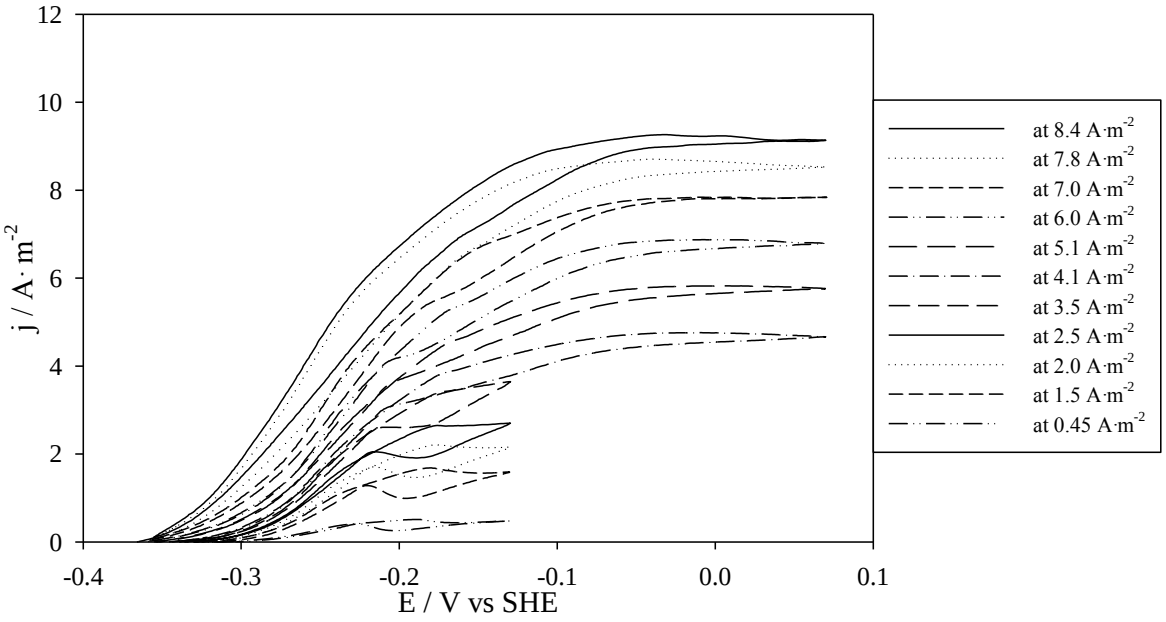

Figure 5.5. Cyclic voltammograms (CVs) monitored for Gold-C reactor at eleven different points of biofilm growth. For each growth-point two CVs replicates were recorded from the anode open circuit potential to 0.07 V (vs. SHE). For the first five growth-points, two CVs replicates were recorded between open circuit potential and -0.13 V (vs. SHE) to preserve the immature biofilm. Only the second CVs replicate were shown

pH Experiment

After the growth of Gold-A anodic biofilm, several experiments were performed to further investigate *Glk. ferrihydriticus* adaptation to pH changes. CVs were recorded in duplicate for five different pH values (9.3, 9.8, 9.3, 7.9 and finally 9.3), between anode open circuit potential to -0.13 V (vs. SHE) and were normalized to the maximum current density (j_{max}) obtained for each CV. The forward curves of second duplicates CVs are shown in Figure 5.6. These results show that pH variations can shift j/j_{max} curves from left (highest pH) to right (lowest pH). This shift was due to a change of the activation losses which were the lowest when the medium had pH 9.8 and the highest when medium had pH 7.9. That resulted in a consequent highest (at pH 9.8) or lowest (at pH 7.9) current density versus the same anode applied potential. When Gold-A was operated again at pH 9.3 the performances were similar than the values obtained previously at pH 9.3. These results suggested that the changes of activation losses were not due to damage or a conformational change in the bacteria cells and biofilm, but that the performance changes were only due to the changes of the environment conditions, so in the ability of the biofilm to exchange electrons with the anode. Thus, the highest pH in this condition was the most favorable scenario for these bacteria.

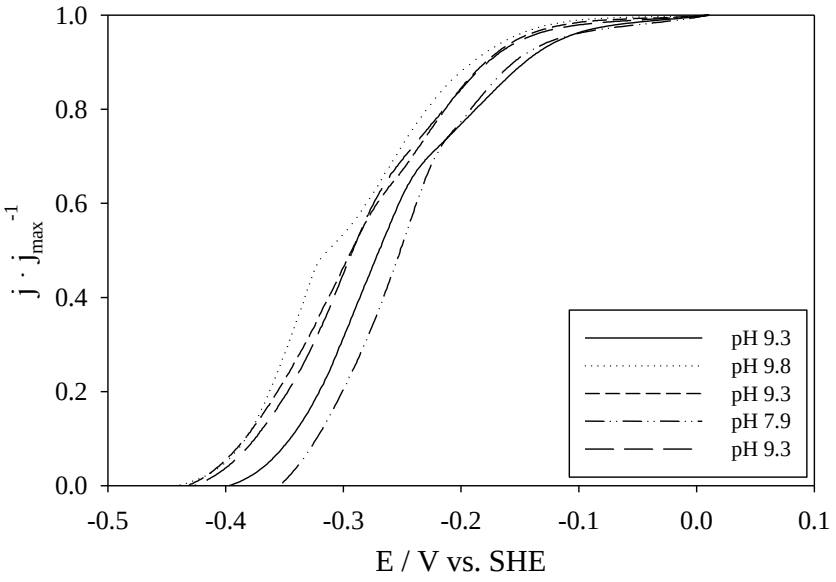

Figure 5.6 shows $j \cdot j_{max}^{-1}$ curves obtained for five different pH points: 9.3, 9.8, 9.3, 7.9 and 9.3.

5.2. Bioelectrochemical electrical current and hydrogen production under alkaline conditions

5.2.1. Specific experimental procedures for section 5.2

Mixed culture inoculation

Anaerobic sludge from a local municipal wastewater treatment plant was inoculated in an i-MFC which was operated for three months in fed-batch mode. Acetate was added every 5-6 days to obtain around 1 g/L in the cell, and half of the medium was replaced by fresh medium every month. pH was manually controlled at 8.5 by adding NaOH to pre-select a population able to survive in an alkaline environment.

Alkaline c-MXCs operation

Alkaline c-MFCs (R_{ext}= 220 Ω) were inoculated by mixing (1:1) used media from the i-MFC and fresh media. Alkaline c-MFCs were converted into alkaline cube-MECs (c-MEC) by avoiding oxygen presence in the cathode with a methacrylate cover and applying a constant potential of 0.8V, as detailed in Chapter 2. Alkaline c-MXCs were operated in batch mode using the basic medium previously described, with the addition of 50mM of 2-bromoethanesulfonate (BES) and fed with approximately 30mM of acetate.

Alkaline Medium

The MXCs alkaline medium (pH= 9.3) was made as previously described in Chapter 2, but 100 mM PBS was substituted by 100 mM K_2HPO_4 solution

Online pH control

The pH was monitored on-line in cube-MXCs by using pH probes (Crison pH electrode 5233) connected to a pH meter (Crison MultiMeter 44) as described in Ruiz et al. [87] (Figure 5.7). The pH was controlled with base (NaOH stock solutions 3 M) dosage by using an automatic burette (Crison MultiBurette 2S). The glass tubes located at the top of the cell allowed the volume of the reactor to be increased. All experiments with online pH control were performed with orbital agitation at 100 rpm (DOS-20L ELMY Sky Line digital orbital shaker) to ensure liquid homogeneity.

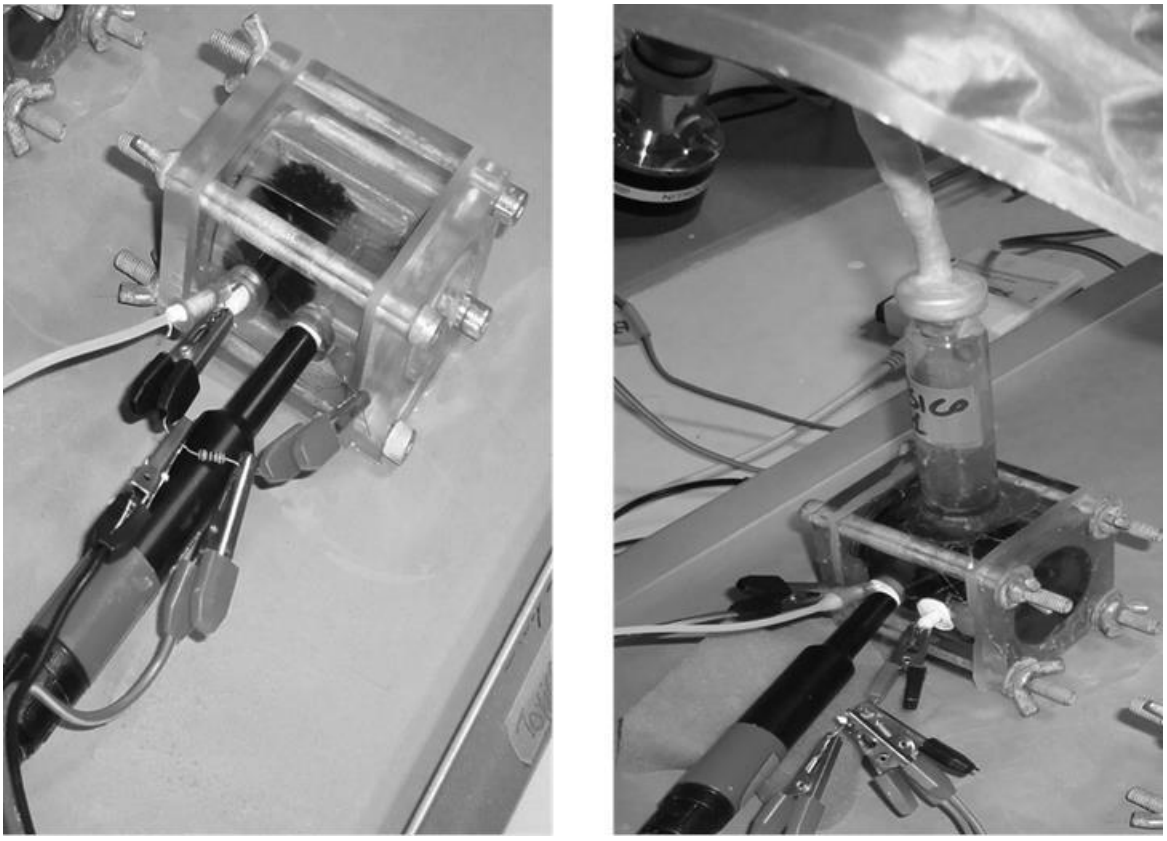

Figure 5.7. Image of an alkaline c-MFC (left) and an alkaline c-MEC (right) with online pH control.

Neutral c-MXCs operation

To compare the results, a neutral c-MFC was built as previously described and inoculated by mixing 1:1 the effluent from an already working neutral i-MFC and fresh medium. Neutral i-MFC and neutral c-MFC were performed as alkaline i-MFC and alkaline c-MFC by using pH=7.3 medium that contained 100 mM PBS buffer with the same components in 1 L of deionized water described before. Neutral c-MFC was also converted into neutral c-MEC like previous described.

Electrochemical analyses

The chronoamperometric method was used to compare alkaline and neutral MFCs performances. The anode was the working electrode and the cathode was used as both the auxiliary and the reference one. Similar chronoamperometric measurements were used to build polarization and power curves for MFCs. In this case, the anode potential (vs cathode potential) was set at 11 levels from -675 mV (anode open circuit potential) to -200 mV. Power curves were calculated from the polarization curves as the product between potential and intensity.

DNA extraction

Samples were obtained from an alkaline MFC and from an alkaline MEC anodes after respectively 90 and 145 days of complete operation. The anode graphite fibers were rinsed with 1 ml of sterile MilliQ water to remove residues from the growth medium or residues from biofilm and then were cut and combined for DNA extraction.

Total DNA was extracted from approximately 0.15 g of wet samples using a PowerBiofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer instructions. Quality and quantity of the DNA was measured using a NanoDrop®

spectrophotometer (ThermoScientific). DNA was visualized under UV in a 0.7% gel electrophoresis with TBE 0.5X (Tris-Borate 50 mM; EDTA 0.1 mM; pH 7.5-8).

High-throughput 16S rRNA gene pyrosequencing

Pyrosequencing was performed as described in section 4.1 with an average of 3000 reads/assay and by using the same couple of primers of the bacterial 16S rRNA gene 338F-907R. Sequences were checked using Uchime [136].

5.2.2. Results and discussion

An alkaliphilic exoelectrogenic bioanode, able to operate at pH higher than 9, was enriched from conventional anaerobic sludge in a two-step procedure to avoid a rough pH change. First, an i-MFC was inoculated with anaerobic sludge from a local municipal wastewater treatment plant and operated for three months in fed-batch mode under "moderate" alkaline conditions (i.e. pH was daily adjusted to 8.5). Acetate (up to 1 g/L in the cell) was added when intensity dropped down. Half of the medium was replaced by fresh medium every month to prevent the shortage of micro- or macronutrients in the medium. After this first enrichment period, an alkaline c-MFC was inoculated with this enriched alkaline microbial community and was, in turn, operated for three months with a controlled pH of 9.3. Figure 5.8 presents the experimental current density profiles obtained during this period. The cell showed initial exoelectrogenic activity but with low intensity values around 1 $mA \cdot m^{-2}$ during the three first batch cycles (20 days). However, a high increase of activity was observed in the fourth cycle because of the anode adaptation to the new operational conditions at alkaline conditions (i.e. pH=9.3). The system showed a reliable performance for more than two months and stabilized current intensity around 9.5 $mA \cdot m^{-2}$ under non-limiting substrate concentrations.

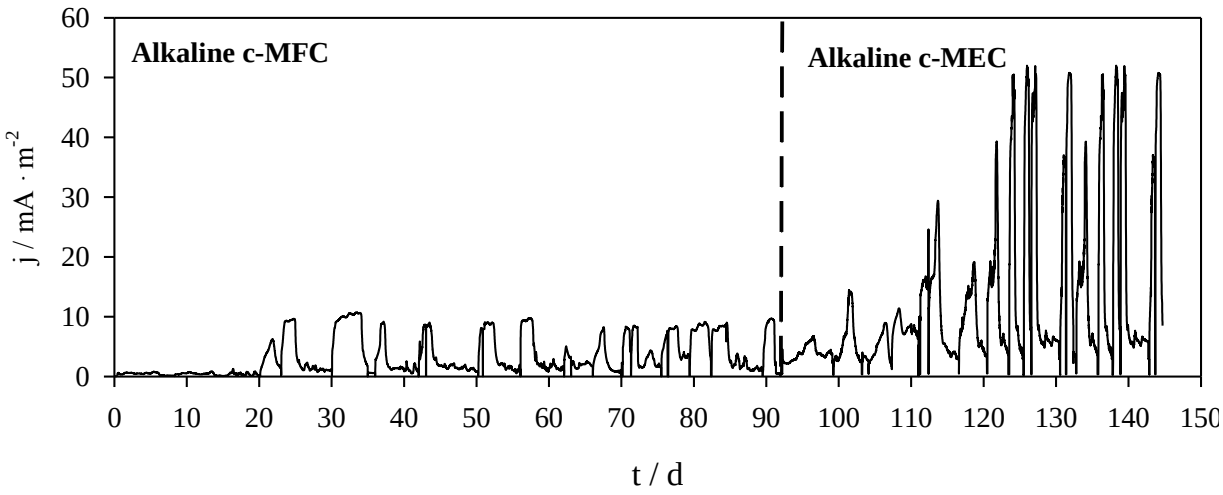

Figure 5.8. Current density profiles for alkaline c-MFC and c-MEC during the operational period.

A parallel c-MFC at neutral pH was inoculated by mixing (1:1) the effluent from an already working i-MFC (at neutral pH) and fresh medium at neutral pH (7.3). This neutral c-MFC was operated for three months and the current intensity achieved was around 9 $mA \cdot m^{-2}$. The CE for the alkaline c-MFC was 60±5% (n=3), which is higher than that obtained with the neutral c-MFC (43±10%, n=3). Similar MFC configurations at neutral pH in the literature have reported lower CE values [91,137,138]. At first glance, this suggests less competition for substrate or, in other words, a more selective environment for exoelectrogens at this pH.

The performance of the alkaline c-MFC was further evaluated on day 70 through polarization and power curves (Figure 5.9). The maximum power (511 μW) for the alkaline c-MFC was obtained with an external resistance of around 100 Ω which was close to the resistance used under normal operation for this cell. This power was slightly lower than the one observed for the neutral c-MFC (618 μW) despite the profiles were very similar.

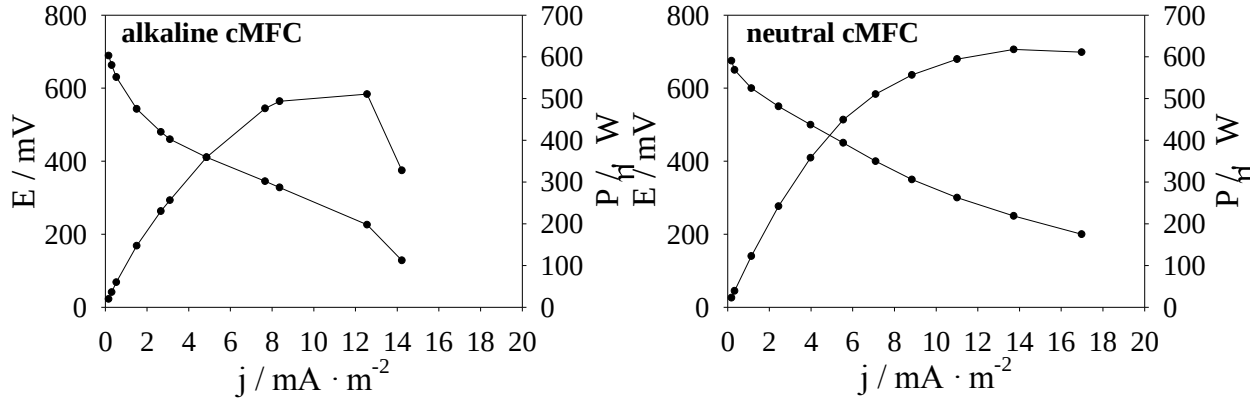

Figure 5.9. Power and polarization curves for the alkaline and neutral c-MFCs

DNA was extracted from the anode of the alkaline c-MFC (day 90) and the microbial community was analyzed using pyrosequenciation (Figure 5.10). The dominant bacterial genus identified was *Alkalibacter* (37% of the total of the sequences) which, to the best of our knowledge, has not been described as an exoelectrogen yet. Nevertheless, we consider *Alkalibacter* sp. as a potential exoelectrogen because of its high abundance in this c-MFC. *Alkalibacter* genus is strictly anaerobic and an alkaliphilic genus of *Firmicutes* phylum [139]. Many genera and species from *Firmicutes* phylum, often from C*lostridiales* order, showed exoelectrogenic activity [25,140]. In this context, *Alkalibacter* genus was never detected before in any MFC fed with acetate [49,55,108] or different substrates [46,47], operated under neutral conditions. A high percentage of *Geoalkalibacter* genus was also detected in the alkaline c-MFC cell (21% of sequences). *Geoalkalibacter* is a genus of the *Geobacteraceae* family that is recognized as an exoelectrogenic genus detected at high operational pH [24]. In section 5.1 and in Badalamenti et al. [24], pure culture MECs were inoculated with *Geolkalibacter ferrihydriticus* and operated, under alkaline condition, as an exoelectrogenic microorganism. The results obtained in the c-MFC are, as far to our knowledge, the highest enrichment for this genus reported in the literature for mixed cultures in alkaline bioelectrochemical systems. Then, operation at alkaline conditions seems a promising selective environment for this genus. On the other hand, *Geobacter* was not detected in the alkaline c-MFC while high values of *Geobacter* (around 70 %) are normally present in neutral MFCs [108].

Acetobacterium sp. was also present (3%). This genus was already detected in bioelectrochemical systems [49,55,108] as an homoacetogenic genus, able to use H_2 as electron donor and CO_2 as electron acceptor to produce acetic acid [106]. Other classified genera of *Clostridiales* order detected were *Proteiniclasticum* sp. (2%) and *Anoxynatronum* sp. (1%). Species of the *Anoxynatronum* genus (1%) are known to be true alkaliphilics with a growth range from pH 7.1 to pH 10.1 and optimal pH for growth at pH 9.1 [139]. *Nitrincola sp.*, which represents 2% of total reads, is also recognized as an alkaliphilic genus. Unclassified family of *Clostridiales* order represents 2% of the bacteria in the anode. Unclassified microorganisms of the phylum *Bacteroidetes* were also found (6%). Their presence is frequent in natural environments and has been reported in anaerobic digestion sludge [141], which was our initial inoculum. Almost the 9% of the sequences could not be ascribed to particular phyla. 10% of the sequences were included in the "others" category as its representation was below 1% of the total reads.

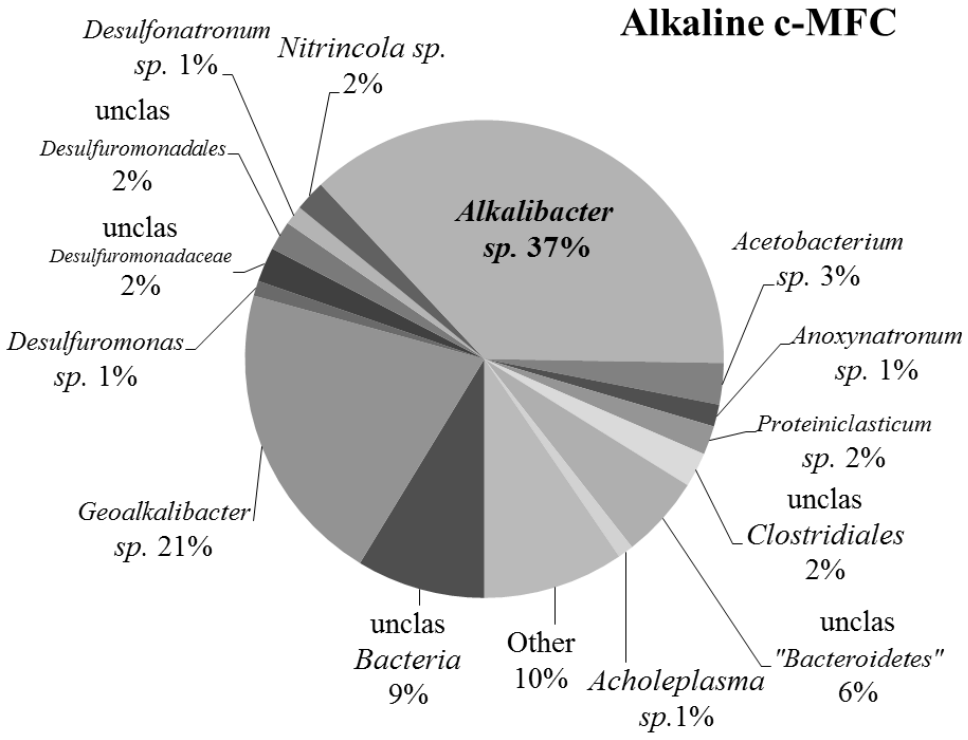

Figure 5.10. 16S rRNA gene pyrosequencing results of anodic alkaline c-MFC bacterial communities.

After 90 days of MFC operation, both cells (alkaline and neutral) were moved to MEC configuration. Current intensity, CE and H_2 production were monitored for both alkaline and neutral c-MEC. Figure 5.8 shows the current density profiles obtained under alkaline c-MEC operation. The exoelectrogenic activity in the alkaline c-MEC increased after 35 days of MEC operation and was maintained stable for one month with high current intensity of around 50 $mA \cdot m^{-2}$ for all batch cycles. On the other hand, the maximum current intensity obtained in the neutral c-MEC was only slightly higher than 30 $mA \cdot m^{-2}$ (Figure 5.11).

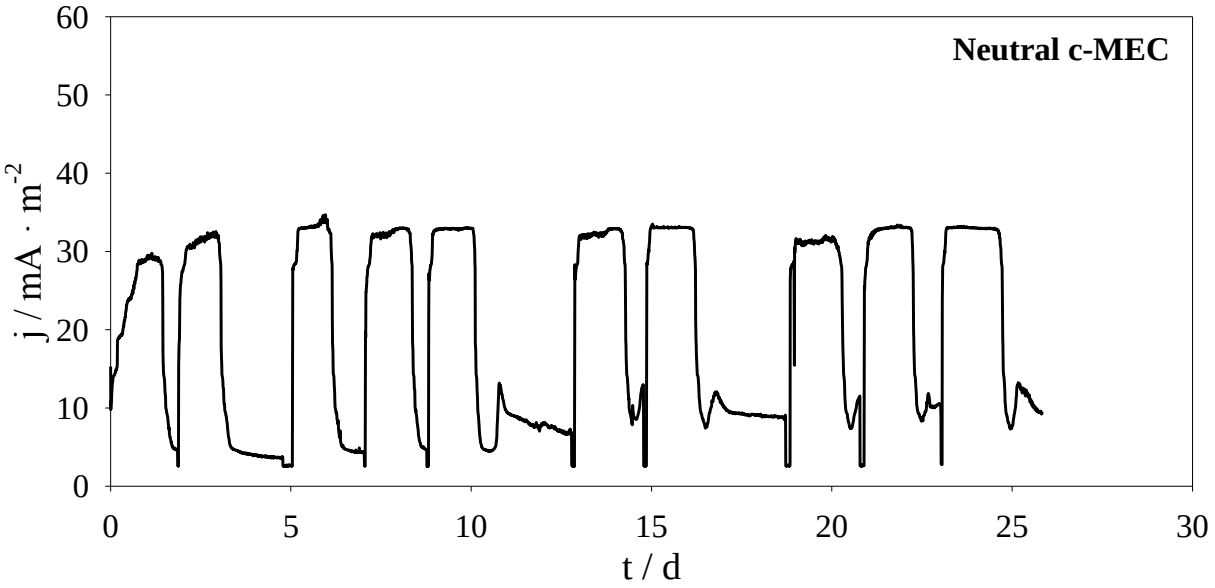

Figure 5.11. Last 25 days of neutral c-MEC operation.

In terms of H_2 production, the best alkaline c-MEC performance was obtained after around 140 days with 2.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$. On the other hand, H_2 production in neutral c-MEC was 1.2 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$. The cathodic efficiency in both cells was close to maximum (around 100%). The CE was similar for both cells: 107±5% (n=2) for alkaline c-MEC and 102±12% (n=2) for neutral c-MEC. Obtaining a CE higher than 100% is often observed in single-chamber MEC and attributed to the presence of homoacetogens as hydrogen scavengers. Part of the hydrogen produced in the cathode is used as electron donor for acetate production which, in turn, is used in

the anode by exoelectrogens. This hydrogen recycling biases the calculation of conventional MEC indicators as CE [38] and a thorough electron balance is required to fully understand the fate of electrons. The presence of homoacetogens was confirmed in the pyrosequencing analysis described below (Figure 5.12).

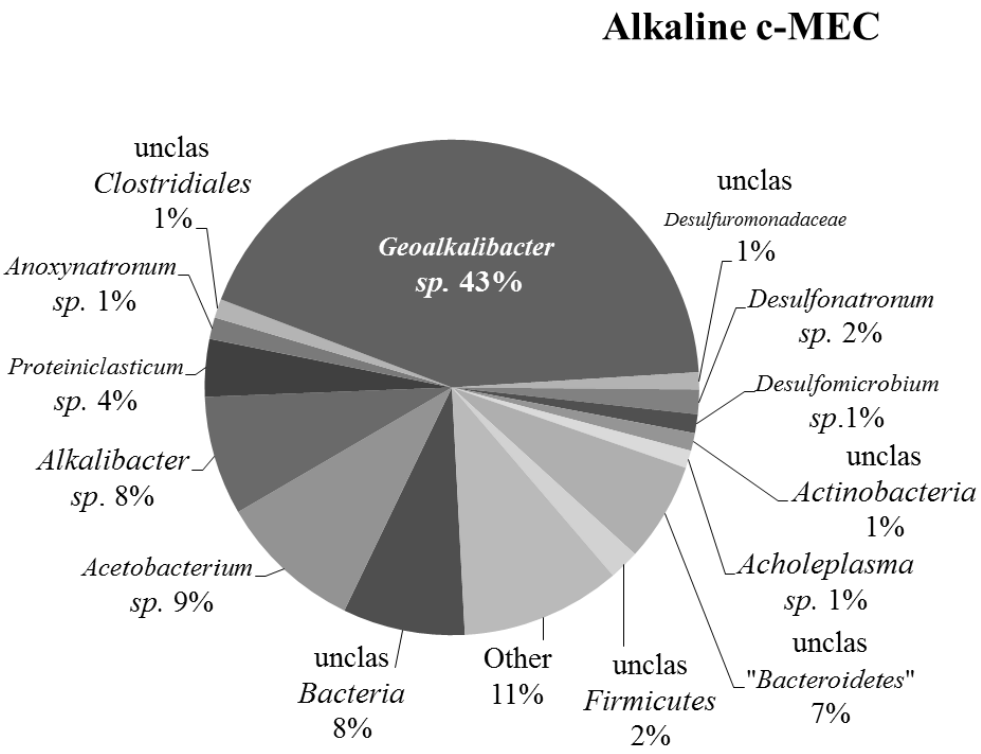

Figure 5.12. Anodic alkaline c-MEC bacterial community analysis by using 16S rDNA 454-pyrosequencing

DNA was extracted from the anode of the alkaline c-MEC at day 145 for a pyrosequencing analysis (Figure 5.12). The dominant bacterial genus identified was *Geoalkalibacter* sp. (43%). When compared to the alkaline MFC scenario, a high increase of *Geoalkalibacter* genus (from 23 to 43%) and a high decrease of the *Alkalibacter* genus (from 37 to 8%) were observed. The *Geoalkalibacter* increase may be linked to the high current density increase observed between the two scenarios (up to six-fold higher). The applied potential may have created a positive

selection force for the *Geoalkalibacter* genus. Further research is required at this stage to understand the interactions between *Geoalkalibacter* and *Alkalibacter* genera.

Acetobacterium genus was more abundant in c-MEC (9%) than in c-MFC (3%), in agreement with the fact that the hydrogen was formed in the cathode and used as electron donor for homoacetogenesis. Although *Acetobacterium* sp. presence prejudices alkaline c-MEC, which showed a CE higher than 100% meaning that part of the H_2 produced was recycled [38], the cell showed a good performance. The rest of the variations of the bacterial community between alkaline c-MFC and c-MEC scenarios were not relevant.

The results of this study demonstrate the selection, enrichment and operation of a totally efficient anodic biofilm operating at high pH. The anodic biofilm was mainly enriched with alkaliphilic bacteria as demonstrated by using pyrosequencing analysis, such as *Geoalkalibacter* sp., with exoelectrogenic activity detected in previous studies, and *Alkalibacter* sp. which was identified in an MXCs for first time in this study. Future studies can confirm the exoelectrogenic activity of species of *Alkalibacter* genus.

5.3. Conclusions

This chapter evaluated the bioelectrochemical production of current and hydrogen under alkaline conditions performed by alkaliphilic ARBs. High current density was obtained in *Glk. ferrihydriticus* pure culture MEC in section 5.1. The study demonstrated the ability of this bacteria specie to generate current density at different alkaline pH, between 7.9 and 9.8. Two different applied potentials were used (+0.07 and -0.13 V). The current density obtained was more stable when the anode applied potential was +0.07 V. The pH experiments showed that *Glk. ferrihydriticus* biofilm was working better at pH=9.8. Further studies are needed to understand the electron transfer mechanism that is used by *Glk. ferrihydriticus* and, moreover, the better applied potential for this specie.

In section 5.2, alkaliphilic ARBs were selected from anaerobic sludge by operating batch reactors at high pH. MXCs inoculated with this culture showed a high *Geoalkalibacter* sp. content in the anodic biofilms, which is a known responsible of exoelectrogenic activity.

Alkalibacter sp. was detected in MFCs and was recognized as a new potential ARB. Alkaline MFC performance was reasonable if compared with a conventional MFC operated at neutral pH. The H_2 production was better in the alkaline MEC than that obtained in the neutral pH MEC. The results in this study also demonstrated that alkaliphilic ARBs are capable to live and perform at high pH as well as the common ARBs as *Geobacter*. This study also underlines the importance of metagenomics study of biofilm growth under different inoculum and operational condition to identify novel ARBs, which can permit its use in different real environments.

CHAPTER 6

Cheese whey in MECs: a suitable substrate for hydrogen production without methane

Motivation

Simple fermentation products are the most common substrates used in BES due to the fact that ARB have heterotrophic metabolism and, thus, they are capable to utilize easy biodegradable substrates as acetate or glucose.

However, several studies showed the use of MXCs-fed by complex substrates as for example starch [46,142,143], cellulose [144,145], propionate, butyrate and glucose [146], lactate [147], xylose [148], oxalate [149], glycerol [46,150], methanol [47], or even with domestic, municipal [151], brewery [152,153], chocolate industry [154], paper recycling [155] biodiesel [156], dairy industry [157–159] and mixed wastewaters [160]. In these systems, bacteria fermentative consortia were capable to degrade complex substrate to obtain simplest substrates, such as acetate which is used by ARB to produce current intensity (in MFC) or H_2 (in MEC).

The use of MXCs to decrease the high chemical oxygen demand (COD) of dairy wastewater is interesting due to the possibility to use the high presence of complex organics to produce energy. 1–5 L of wastewater is produced per liter of milk processed in dairy plants [161] and it needs treatment before to be discharged into the environment.

Several studies showed current intensity production from MFCs fed with dairy wastewater [157,158]. The use of dairy wastewater for H_2 production in MEC, was suggested in Montpart et al. [46] due to the absence of methanogenesis in a long term MEC operated with synthetic medium and fed with powder milk. In Moreno et al. [159] H_2 was produced from cheese whey in a two-stage process, which include dark fermentation and MEC. In both studies no methanogenic activity was shown without using chemical inhibitors.

The aim of this study was the H_2 production from cheese whey, by using a single-chamber MEC inoculated with a syntrophic consortium consisting of cheese whey fermenting bacteria and ARB. Pyrosequencing analyses were conducted during the study to investigate the microbiological community under different operational conditions.

6.1. Specific experimental procedures

A consortium able to degrade cheese whey was obtained by separately growing the fermentative community in a culture flask and ARB microbial community in an acetate-fed c-MFC (ac-MFC). Next, fermentative consortium was joined in working i-MFC. Once it was ensured that a syntrophic consortium had developed in cheese whey-fed c-MFC (cw-MFC), the biologically enriched anode was moved to a c-MEC (from now called cw-MEC), which was also fed with whey in batch cycles and fed-batch. In the final part of the study, medium was substituted for 100 mM of PBS distilled water solution, without addition of supplementary compounds.

Cheese whey (obtained from "Centre Especial d'Investigació Planta de Tecnologia dels Aliments" of Universitat Autonoma de Barcelona) had a high organic matter content (74.8 g/L of COD) and a pH of 6.3.

Culture flask

The culture flask was a 100 mL glass bottle tightly capped with PTFE rubber septum and an aluminum crimp on top. It was magnetically stirred and kept in a 37ºC room. The bottle was filled with 50 ml of anaerobic digester sludge (municipal wastewater treatment plant of Manresa, Catalonia), milk whey (4 g/L of COD), 50 mM of BES and deionized water up to 70 ml.

The culture flask was operated under fed-batch mode with cycles of around seven days duration. Every time the system was fed, the mixed liquor was centrifuged (4 min at 5000 rpm) to enhance biomass retention, the supernatant medium was discarded, and the sludge was then resuspended up to 70 ml in deionaized water containing whey (4 g/L of DQO) and 50 mM of BES. Before closing the bottles, nitrogen was sparged to ensure anaerobic conditions. Volatile fatty acids (VFAs) and chemical oxygen demand (COD) were measured to assess the development of the fermenting community and gas analyses from the headspace allowed to ensure that no methane was being produced.

Electrochemical calculations

CE was calculated using equation 6.1.

$$CE = \frac{M_{O_2} \int_{t_0}^{t_F} Idt}{F \cdot b_{O_2} \cdot V_L \cdot \Delta COD} \qquad (6.1)$$

where t_0 and t_F are the initial and final times of a batch experiment, I is the current intensity, M_{O2} is the molecular weight of the oxygen, F is the Faraday's constant (96485 C/mol e^-), b_{O2} is the number of electrons exchanged per mole of oxygen, ΔCOD is the COD concentration change over a batch cycle, and V_L is the volume of liquid in the reactor.

Chemical Oxygen Demand

Chemical oxygen demand (COD) was analyzed by commercial test tubes LCK 714 100-600 mg $O_2 \cdot L^{-1}$ (Hach Lange) according to the manufacturer's instructions. The change in dichromate concentration was measured colorimetrically by using the spectrophotometer VIS DR 2800 (Hach Lange).

Electrochemical analyses

Electrochemical analyses were performed to evaluate MEC by using a Multi Autolab system (Ecochemie, Utrecht, Netherlands) by means of a linear scan voltammetry. The anode was the working electrode and the cathode was used as both the auxiliary and the reference one. LSV was recorded from around 0.2 V to 1.0 V at a scan rate of 1 mV/s.

DNA extraction

Samples were obtained from cw-MEC anode at day 80, before the change in the operating conditions and at the end of a fed-batch cycle (after 110 days of total operation). Total DNA was

extracted from approximately 0.15 g of wet samples using a PowerBiofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) as previously described in section 5.2

High-throughput 16S rRNA gene pyrosequencing

Pyrosequencing was performed as described in section 4.2 with an average of 3000 reads/assay and by using the same couple of primers of the bacterial 16S rRNA gene: 357F-926R. Sequences were checked using Uchime [136].

6.2. Results and discussion

The first step in this study was the selection of a syntrophic anodic consortium with exoelectrogenic capability. The consortium comprised fermentative bacteria to degrade the complex substrates from cheese whey to VFA and ARB that lived off the VFA produced. Both bacterial communities were selected in parallel in different reactors. The cheese whey fermentative community was selected from anaerobic sludge using the anaerobic culture flasks (37ºC) amended with a BES to suppress the last step of anaerobic degradation (i.e. VFA to methane). After 10 days of anaerobic selection, cheese whey was successfully degraded and converted to VFA. The composition of the gases accumulated in the headspace of the culture flask showed a significant H_2 production due to fermentation and no methane accumulation.

In parallel, an acetate-fed MFC (ac-MFC) was inoculated with ARB from an already running i-MFC of our lab following common guidelines [48]. Figure 6.1 shows the current density of the last two cycles of the ac-MFC operation. This ac-MFC was bioaugmented at day 0 with the previously selected fermentative bacteria to have a full consortium able to use cheese whey as substrate for exoelectrogenesis. Sludge from the anaerobic cheese-whey degrading culture flask mixed with fresh medium (1:1) was used for the bioaugmentation. Then, day 0 in Figure 6.1 stands for the first cycle with the whole fermentative consortium ac-MFC and fed with cheese whey, becoming, thus, cheese whey-fed MFC (cw-MFC). cw-MFC was operated for around 45 days (Figure 6.1). After 25 days of operation, the current density reached a similar value to that

in ac-MFC. VFAs were not detected at the beginning or at the end of each batch cycle indicating that they were simultaneously produced and consumed due to the syntrophic activity of the consortium. The coulombic efficiency in this period was 49±8% (n=2), which is a reasonably good value considering that cheese-whey is probably not directly used by ARB and an intermediary biological step is required. Table 6.1 compares the CE previously obtained in different studies with different substrates. The cw-MFC performance was really similar to the CE obtained using milk powder in Montpart et al. [46] study. Far better performance was obtained in cw-MFC if compared with other MFC fed with dairy wastewater (dairy WW) presented in other previous works [157,158]. Moreover, cw-MFC CE was slightly higher than the CE obtained with neutral acetate-fed c-MFC (43±10%, n=3) shown in section 5.2.

Table 6.1. Comparison of CE obtained in different MFC studies using substrates similar to cheese whey.

	Experimental setup	MFC CE	References
Acetate	Alkaline MFC	60±5 %	Section 5.2
	Neutral MFC	43±10 %	Section 5.2
Complex Substrates (Dairy products)	Milk	52±6 %	[46]
	Dairy WW	27 %	[158]
	Dairy WW	17 %	[157]
	Cheese whey	48±8 %	This study

After this period, the cw-MFC anode was moved to a MEC cell and BES was added to avoid methanogenesis. The MEC was operated with cheese-whey as sole substrate for around 35 days (from here onwards, cw-MEC). The maximum current density (Figure 6.1) increased up to four times higher than that in ac-MFC or cw-MFC after only four cycles (approximately 25 days).

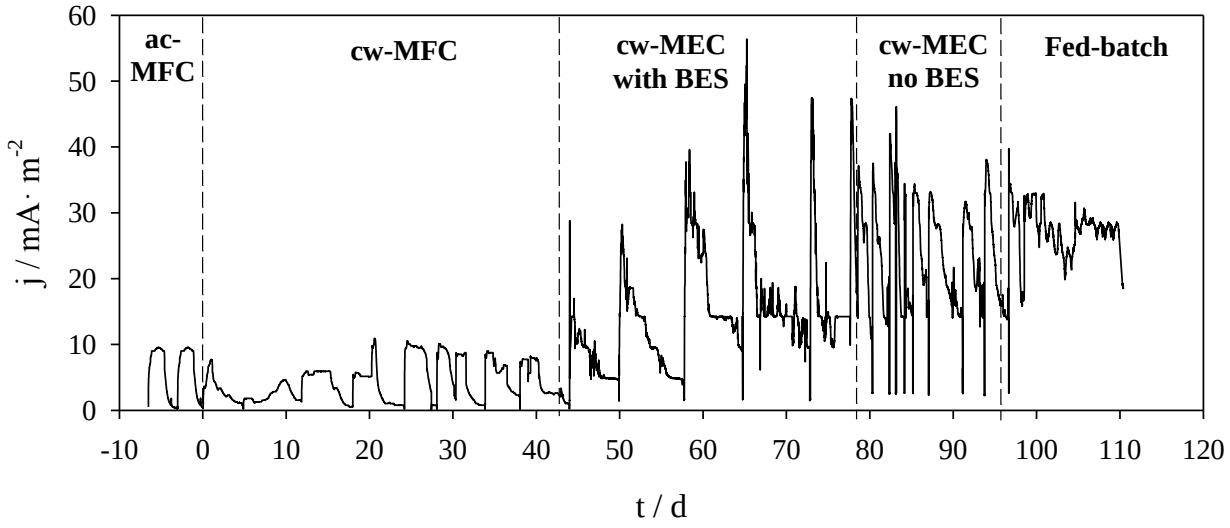

Figure 6.1. Current density (mA · m^{-2}) monitored during the complete operational period. The graphic is divided into five periods: i) last two cycles of ac-MFC, ii) cw-MFC operation, iii) cw-MEC operation with BES and synthetic medium, iv) cw-MEC operation without BES, diluted cheese-whey (2 g/L COD) and PBS (100 mM) and v) fed-batch period.

One of the hypothetical benefits in the use of dairy products as substrate for bioelectrochemical systems is the potential minimization of methanogenic activity. According to previous works [46,159], methanogens do not easily outcompete exoelectrogens in these systems in spite of the favorable conditions (i.e. anaerobic environments with biodegradable COD). In the first period of the cw-MEC operation, a methanogenic inhibitor was added in the medium and the system performed successfully 0.8 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$ with a 49±2% (n=2) of cathodic efficiency.

However, we aimed at gaining more insight on the competition between methanogens and exoelectrogens in this scenario. Thus, at day 80,cw-MEC the methanogenic inhibitor and the micro- or macronutrients were removed from the medium and the cell was operated under conditions closer to real wastewater using diluted cheese-whey (down to 2 g/L COD) only amended with PBS (100 mM) to avoid pH changes.

Figure 6.2 shows these two sequential batch cycles obtained in the cw-MEC under both conditions (i.e. with and without BES plus micro- and macronutrients). Although the current density obtained with BES and full medium was slightly higher probably due to the micro and macro nutrients that could improve biomass activity, both profiles were very similar.

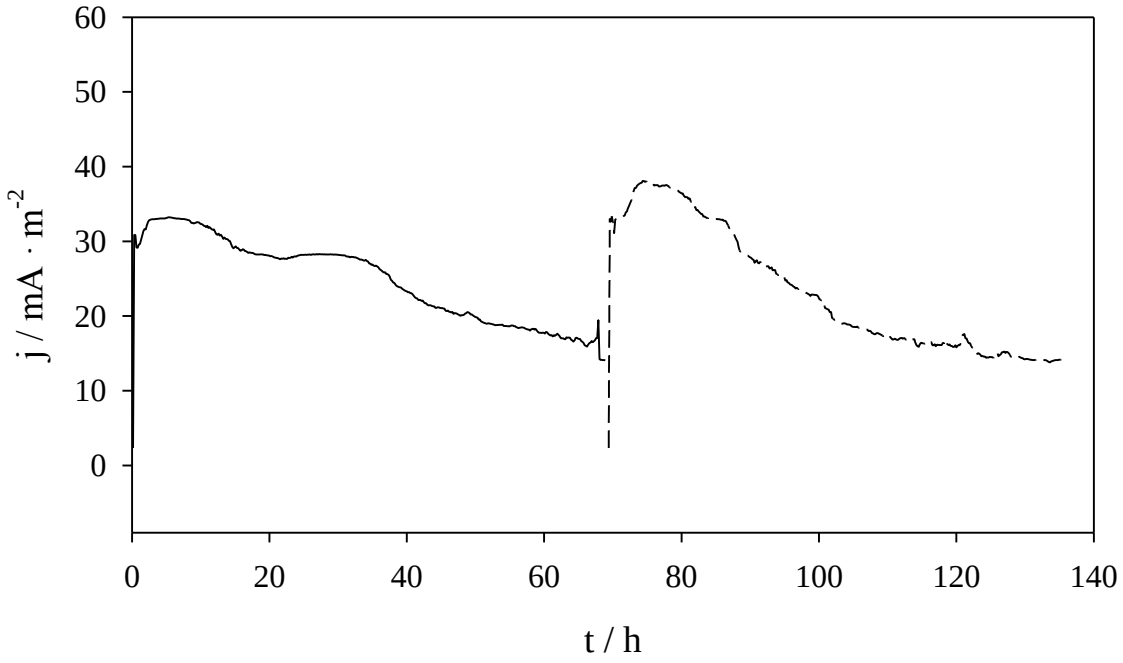

Figure 6.2. Comparison between two MEC batch cycles: diluted cheese-whey with PBS (solid line) and diluted cheese-whey and full medium (dashed).

The average coulombic efficiency in the full MEC period was around 120% independently of the BES presence, suggesting that methanogens were not present in the cell. This was also confirmed by gas analysis. The average H_2 production in both periods was around 0.6 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$ with a 35% of cathodic efficiency. High coulombic efficiency linked to low cathodic efficiency indicates the occurrence of H_2-recycling due to homoacetogenic bacteria [38]. Homoacetogenic bacteria were previously found in cathodic biofilms of MEC when the hydrogen retention rate was high (section 4.1).

A sample for pyrosequencing of the anodic community was withdrawn at day 80. The analysis (Figure 6.3) showed a high presence of *Geobacter* sp. (30% of the total of the sequences), which is the most usual ARB found in acetate-fed MXCs [27,93,162,163]. *Geobacter* genus was, thus, responsible of exoelectrogenic activity in the system. *Enterococcus* sp. (14% of sequences) is a lactic acid bacteria that has important implications in the dairy industry [164]. Different species of enterococci are found in dairy products, as *E. faecalis* and *E. faecium* [164,165]. Unclassified *Bacteroidetes* accounted for 18% of the total. They are often present in MXCs inoculated with anaerobic sludge [46,49] and they are known to be degraders of high molecular weight organic matter like proteins and carbohydrates. 5% of total reads were unclassified *Deltaproteobacteria* class that include *Geobacter* genus. *Sphaerochaeta* sp. (2%) include bacteria that are capable to fermentate carbohydrates including pentose and hexose monosaccharides, disaccharides and soluble starch to obtain products of glucose fermentation including acetate, formate and ethanol [166].

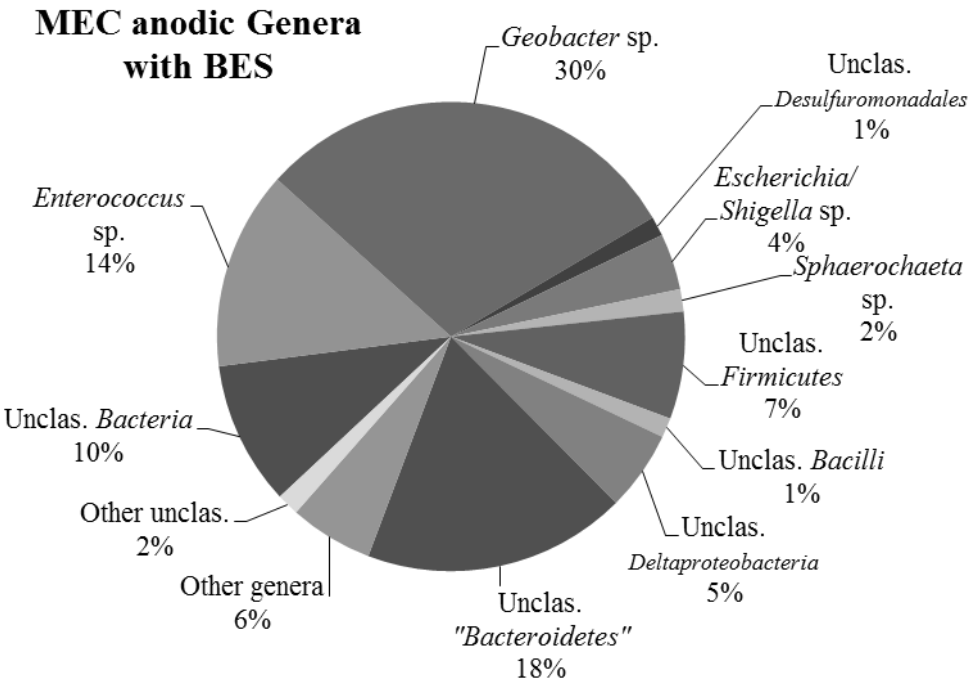

Figure 6.3. Genera of cw-MEC distribution in the anodic biofilm obtained by pyrosequencing. Sequences accounting for less than 1% of the total reads have been included in the "Other genera" category.

Two linear sweep voltammetries, LSVs, (with acetate and with cheese-whey) were completed to gain more insight into the performance of syntrophic consortium in the MEC (Figure 6.4). The acetate-based current density was only slightly higher, which confirmed that the exoelectrogenic activity was not limited by the fermentation step as previously observed in the transition from ac-MFC to cw-MFC (Figure 6.1, day 25). Moreover, LSVs showed that, at 0.8 V (normal operation applied cell voltage), MEC fed with cheese whey reached a higher current density (around 5% more) than when it was working with acetate.

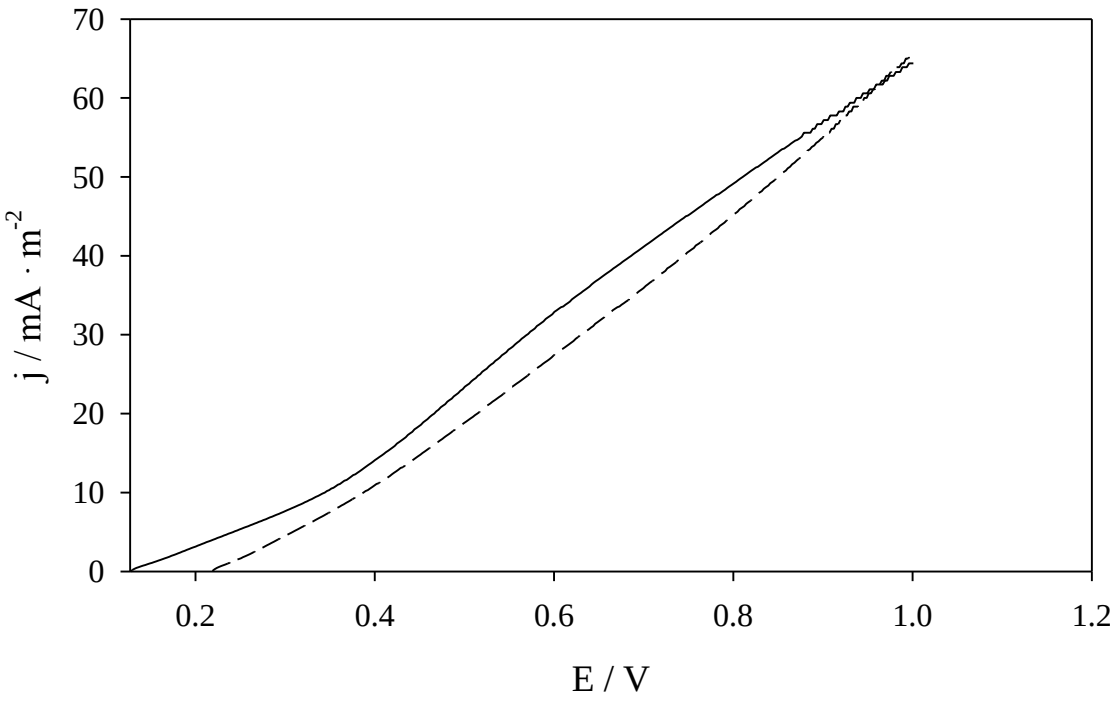

Figure 6.4. MEC performance evaluated by linear scanning voltammetry by feeding the reactor with whey (solid line) and with acetic acid (dashed line).

Methane was not observed in any of the cycles under the cw-MEC operation despite BES was not dosed for more than 20 days. That agrees with the hypothesis of methanogens not being favored when the substrate is related to the dairy industry. However, an extra experiment was planned to test more unfavorable conditions. A fed-batch cycle experiment was conducted to

increase the hydrogen residence time so that methanogenesis was even more favored since hydrogen would be always present in the system. As previously described [44], the increase of residence time favors the growth of methanogens in the system due to high presence of H_2 in the reactor.

The system was periodically spiked with 100 mM PBS water solution of 1 g/L COD of whey. This fed-bacth experiment (Figure 6.5) lasted for more than 15 days and no methane production was measured, which corroborates the apparent intrinsic capacity of milk wastewater fermentation to inhibit methanogenesis [46,159]. In fact, the increase of the hydrogen retention time resulted in an increase of homoacetogenesis due to the high presence of H_2. This observation was confirmed by the increment of CE (170%) and also by the decreasing of H_2 production rate (0.4 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$), due to the H_2-recycling activity.

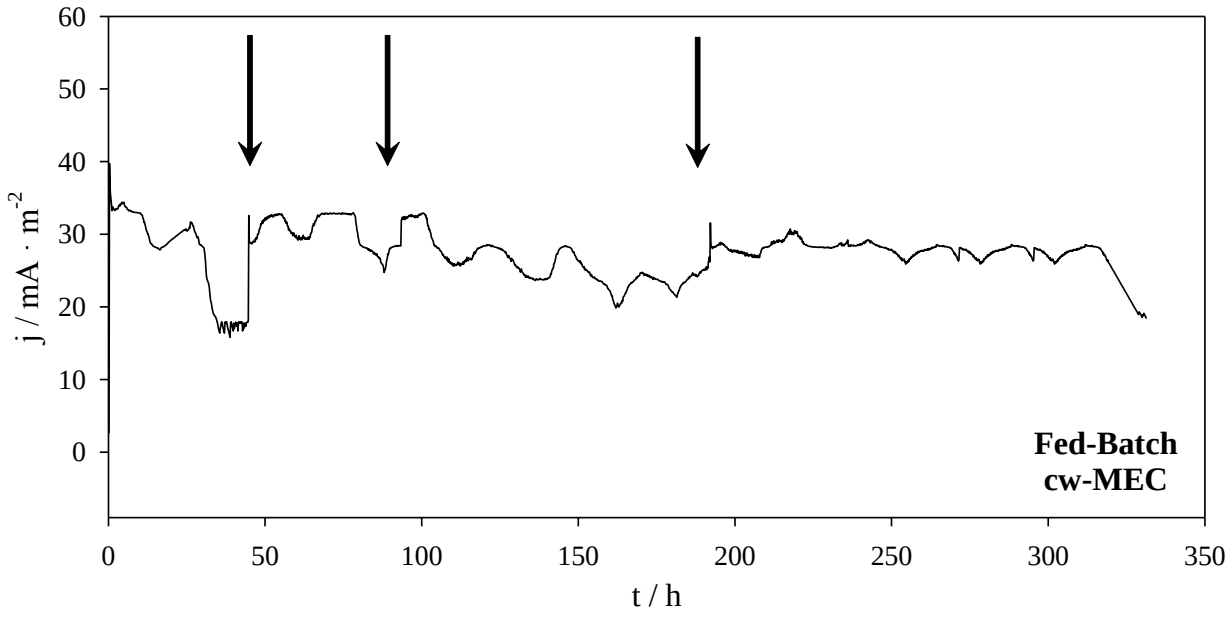

Figure 6.5. Current density profiles for the cw-MEC batch experiment. The arrows indicate the feeding time (45, 94 and 192 hours).

A microbiological sample for pyrosequencing from the anodic biofilm was collected after the fed-batch period (Figure 6.6). An increase in the concentration of sequences of *Geobacter* (37 %) and *Enterococcus* (22 %) genera was detected. *Enterococcus* species exhibit low milk acidifying ability, as previously reported [164]. Species of *Dysgonomonas* genus (2%) were previously found in a MFC fed with rice paddy field soil as a carbon source [167] due to their ability to produce acetate by fermenting glucose, lactose and other carbohydrates [167]. Dairy wastewater showed the capacity to inhibit methanogenesis without the use of a chemical inhibitor, according to previous studies [46,159], even in the worst case scenario (i.e. high hydrogen retention time, fed-batch). The conjunction of high retention time and the intrinsic ability of dairy wastewater to inhibit methanogenesis enhanced H_2-recycling activity, which can undermine H_2 production.

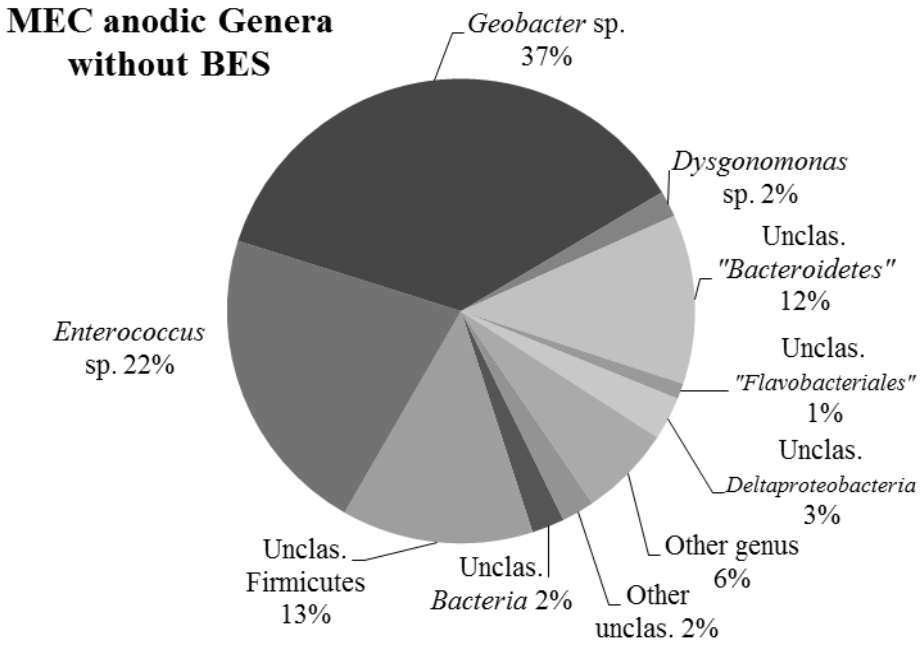

Figure 6.6. Bacterial genera of cw-MEC distribution in the anodic biofilm obtained after one month of operation without BES. Sequences accounting for less than 1% of the total reads have been included in the "Other genus" category.

6.3. Conclusions

This study shows, for the first time, a method to select an efficient syntrophic consortium to produce H_2 directly from cheese-whey in a single-chamber MEC. Cheese-whey was fermented mainly by lactic acid bacteria (*Enterococcus* genus) and other fermenting bacteria as *Sphaerochaeta* and *Dysgonomonas* genera. Exoelectrogenic activity was performed by the common ARB *Geobacter* sp., which used the acetate obtained by fermentation as electron donor.

The CE was 49±8% in cw-MFC, which is a good value considering that cheese-whey is not directly used by ARB and an intermediary biological fermentation is previously required. The CE was higher than the most of the CE reported in previous studies with dairy products fed MFCs.

Cheese-whey syntropic consortium confirmed an intrinsic ability to inhibit methanogenesis also without any external chemical inhibition and increasing the residence time. At the same time, the abundance of proteins and other important compounds permitted the biofilm sustantation without the addition of the micro- or macronutrients generally contained in syntetic lab-scale media.

Therefore, the results obtained in this study demonstrated the pontenciality of whey to be a good carbon source for H_2 production in MEC (0.8 $L_{H2} \cdot L^{-1}_{REACTOR} \cdot d^{-1}$).

CHAPTER 7

General Conclusions

The main conclusions of this thesis are summarised in this section.

The principal aim of this thesis was the microbiological and bioelectrochemical study of the bacterial and *Archea* communities that live in single chamber MXCs in view of better performances. In this frame, the main achievements of this thesis were:

Optimization of 16S gene-targeted metagenomics analysis and quantitative PCR

- The construction and the use of functional qPCR standard curves (chapter 3) permitted the absolute quantification of samples obtained from different reactors. That gave the opportunity to assess the microbiological evolution in time of our reactors.

- The use of 16S rRNA gene 454-pyrosequencing and RDP Pyrosequencing Pipeline tools permitted the metagenomics study of different MXC bacterial or *Archaea* communities including ARB (neutral and alkaline), ARB competitors (e.g. methanogens), fermentative bacteria (chapter 6), BES degrading bacteria (chapter 4) and H_2-recycling homoacetogens, among others.

Improving ARB enrichment protocol of MXC anodes in order to obtain a better MEC performance

- The anodes from MFCs inoculated with low external resistances (chapter 3) showed higher H_2 production rate and gave higher current intensity when transferred and operated as MECs. This was a really important achievement to optimize MXCs inoculation. However, the majority of the MXCs used in this thesis were inoculated by using medium external resistances to obtain the best performance also in MFC configuration. Our experiments showed that the highest power output was obtained from an MFC operated using medium external resistances due to the similartiy between the external resistance and the cell internal resistance.

- qPCR confirmed that the highest content of *Geobacter* in the anodic biofilm was obtained from an MFC inoculated with low external resistance that, in turn, was producing the highest current intensity.

Limitations of the most used methanogenesis inhibitor: 2.bromoethanesulfonate (BES)

- BES could be degraded in MFCs independently of the exoelectrogenic activity.
- BES degradation is a biologic process and pyrosequencing confirmed the presence of a BES-degrading community.
- BES degradation was only observed under an air-cathode configuration. MECs that are operated under strict anaerobic conditions are not affected by this phenomenon.
- Methanogenesis was not totally inhibited in a long-term single chamber MEC, despite the high concentration of BES (100 mM). The inhibition of methanogenesis was obtained only when the MEC was operated using 200 mM of BES concentration (200 mM). This value is much higher than previous results reported in the literature.
- Pyrosequencing analysis and qPCR demonstrated a high presence of hydrogenotrophic methanogens.
- The inhibition of methanogenesis activity resulted in an increase of H_2-recycling. Consequently, a long-term single-chamber MEC where methanogenesis is well inhibited could be undermined by homoacetogenic bacteria.

Obtainment and characterization of alternative electroactive communities selected using high pH inoculum and operational conditions

- The experiment performed with *Geoalkalibacter ferrihydriticus* pure culture showed a high current density production, demonstrating the feasibility of obtaining exoelectrogenic activity at high pH.

- An alkaline electroactive community was obtained in an air-cathode MFC inoculated with anaerobic sludge and operated under alkaline condition (pH 9.3)

- The alkaline MFC showed a better performance when compared with a neutral pH MFC. The alkaline MFC showed a higher CE, which was probably due to the lower competition for substrate, which means a more selective environment for exoelectrogens at this pH.

- H_2-production was higher for an alkaline MEC compared with a neutral pH MEC.

- Pyrosequencing analysis in mixed culture alkaline MXCs confirmed the presence of *Geoalkalibacter* genus.

- The dominant bacterial genus identified in a DNA sample extracted from alkaline MFC anode was *Alkalibacter*. However, its concentration decreased when the same anode was operated in MEC condition.

Selection and characterization of an efficient fermentative-exoelectrogenic community able to produce electricity and H_2 using cheese-whey as substrate

- A cheese whey fermentative-exoelectrogenic community was selected from anaerobic sludge inoculum. Electricity in MFC and H_2 in MEC were produced efficiently by a sintrophic consortium able to ferment cheese-whey to VFAs, which, in turn, were used as electron donors by ARB.

- The abundance of proteins and other important compounds content in cheese whey, permitted the ARB sustantation in MEC without the addition of micro or macro nutrients generally contained in syntetic lab-scale MXCs media.

- Our study confirmed that dairy wastewaters present an intrinsic ability to inhibit methanogenesis without using strategies as the addition of chemicals as BES or physical separation of H_2.

- Pyrosequencing of anodic MXCs results confirmed the presence of lactic acid bacteria, other fermenting bacteria and *Geobacter*

- Cheese-whey is a good candidate to be used as carbon source in MEC.

Future Work

We can only see a short distance ahead, but we can see plenty there that needs to be done.

-Alan Turing-

The results of this thesis suggest a variety of different research directions for future works:

- Methane production in single-chamber MEC provides an alternative to hydrogen production. Lower energy recoveries can be achieved but, in return, the reactor design and operation would be simpler. In this context, the combination of anaerobic digestion and MEC could be explored to reach high purity methane production.
- BES could be employed for outcompeting methanogens and enhancing acetate production in microbial electrosynthesis.That could permit the production of other add-value chemical compound (*i.e.* acetate) that can be transport and manipulate more easily than H_2.
- The use of real alkaline wastewaters or substrates, as crude glycerol, for energy and hydrogen production, seems to be a good strategy to restrict ARB competition, increasing MXC efficiency and decreasing H_2-recycling phenomena.
- Pure culture studies of *Alkalibacter* species, similar to the study performed for *Geoalkalibacter* during this thesis, could confirm the exoelectrogenic activity of this genus.
- Finally, the selection of acidophilic fermentative-exoelectrogenic communities could be employed for the purpose of energy and hydrogen production from dairy wastewaters without the use of buffer. Moreover, the generation of extreme conditions, due to the natural acidification because of lactic fermentation could help to create a more selective environment that can improve the performance as described for alkaline conditions.

Glossary

Acetoclastic methanogens: methanogenic *Archaea* that can use acetate to generate methane

Anode: electrode of an electrochemical device that accepts electrons from an electrochemical reaction.

Anode respiring bacteria (ARB) or exoelectrogenic bacteria or electrogens: bacteria that are able to oxidize organic matter under anaerobic conditions and to transfer the electrons gained in their metabolism out of the cell using a solid electrode (the anode) as electron acceptor.

Applied voltage: voltage provided from an external energy source to drive the reactions in a microbial electrolysis cells

Bioelectrochemical system: a system that combines the metabolism of microorganisms with electrochemistry

Biofilm: multilayered aggregation structure of microorganisms on a solid support.

Cathode: electrode of an electrochemical device where reduction reaction occurs

Cathodic gas recovery (rCAT) or cathodic efficiency: ratio of coulombs consumed in hydrogen production to coulombs arriving to the cathode as current intensity.

Chemical oxygen demand (COD): measure used to indicate the amount of organic material in wastewater. It is expressed in mg O_2/l, which is the amount of oxygen needed to oxidize the entire organic material to carbon dioxide.

Conductive biofilm matrix: polymeric matrix produced by ARB in the biofilm that allows electron transport at a distance from the anode.

Coulombic efficiency (CE): ratio of coulombs circulated in the electrical circuit to coulombs contained in the substrate oxidized.

Cyclic voltammetry (CV): Electrochemical technique to characterize the electron transfer process where the potential of the electrode to study is varied in a ramped linear fashion and the response of the system in current intensity is monitored, I = f (E). The forward and reverse scan on the electrode potential results in a cyclic response of current intensity.

Electroactive microorganisms: microorganisms that are capable of either donating electrons to or accepting electrons from an electrode.

Electromotive force (emf): potential difference between the cathode and the anode, which is positive for a thermodynamically favorable reaction

Extracellular electron transfer: mechanism by which electrochemically active microorganisms donate electrons to or accept electrons from an electrode.

Fermentation: the microbial oxidation–reduction reaction using organic compounds as electron donors and acceptors in the absence of an exogenous electron acceptor.

H_2-recycling: situation caused by syntrophic interactions between ARB, H_2-oxidizing ARB and homoacetogenic bacteria. Hydrogen produced electrochemically at the cathode of MEC can be consumed as electron donor by H_2-oxidizing ARB and it can be consumed to produce acetate by homoacetogenic bacteria, which can be further used as electron donor by ARB. No net hydrogen production and higher current intensity than expected result from this situation.

Hydrogenotrophic methanogens: methanogenic Archaea that can reduce CO2 with H2 to generate methane

Homoacetogenesis: homogeneous microbial formation of acetate using an electron donor such as hydrogen.

Homoacetogenic bacteria or homoacetogens: autotrophic strictly anaerobic bacteria that catalyze the formation of acetate from different substrates

Linear sweep voltammetry (LSV): electrochemical technique used to characterize electron transfer processes in which the potential of the working electrode is changed in a ramped linear fashion and the response of the system in terms of current intensity is monitored.

Methanogenesis: microbial production of methane.

Methanogenic *Archaea* or methanogens: microorganisms that produce methane as a metabolic byproduct in anoxic conditions.

Microbial fuel cell (MFC): bioelectrochemical system that is capable of converting the chemical energy of dissolved organic materials directly into electrical energy.

Microbial electrolysis cell (MEC): bioelectrochemical system that is capable of generating a product (e.g. hydrogen) from dissolved organic materials and that drives the reactions with an electrical energy input.

Nanowire: ARB electroactive pili or pilus-like structures that are used for extracellular electron transfer.

Ohmic loss: voltage loss due to the internal resistance of the bioelectrochemical system, which is generated by the resistance to the flow of electrons in electrodes and connections and the resistance to the flow of ions in the electrolytes and the membrane

Open circuit voltage/potential: voltage that can be measured after some time in the absence of current.

Reference electrode: electrode with stable and well-known electrode potential. Stability is reached by buffered or saturated redox systems that maintain constant concentration of reactants.

Single chamber MXC: bioelectrochemical system which consist in only one chamber where oxidation and reduction reactions occur

Standard hydrogen electrode (SHE): redox electrode which forms the basis of the thermodynamic scale of oxidation-reduction potentials. By convention, it is zero at all temperatures to form a basis for comparison with all other electrode reactions.

Starvation: total lack of nutrients needed for living.

Syntrophy: nutritional association among species, which live off the products of other species.

Two-chamber MXC: bioelectrochemical system with a membrane that physically separates oxidation and reduction reaction products.

List of Acronyms & Abbreviations

A	Ampere
A	Adenine (only in sections 1.10 and 3.1)
Ac-MFC	Acetate-fed Microbial Fuel Cell
ARB	Anode Respiring Bacteria
ARC	Archaea
BES	2-bromoethanesulfonate
bp	Base Pair
C	Citorsine (only in sections 1.10 and 3.1)
c-MEC	Cube MEC
c-MFC	Cube MFC
COD	Chemical Oxygen Demand
CV	Cyclic Voltammetry
cw-MEC	Cheese Whey MEC
cw-MFC	Cheese Whey MFC
DNA	Deoxyribonucleic Acid
E	Potential
e.g.	exempli gratia (for example)
et al.	et alii (and others)
F Primer	Forward Primer
G	Guanine (only in sections 1.10 and 3.1)

Gc	Gas Chromatography
GEO	*Geobacter*
I	Current Intensity
i-MFC	Inoculum MFC
LB	Lysogeny Broth
MBT	Methanobacteriales
MEC	Microbial Electrolysis Cell
MFC	Microbial Fuel Cell
MXC	MFC and MEC
P	Power
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real-Time Polymerase Chain Reaction
PTP	PicoTiterPlate™
R Primer	Reverse Primer
R_{ext}	External Resistance
R_{int}	Internal Resistance
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SEM	Scanning Electron Microscope
SHE	Standard Hydrogen Electrode

t	Time
T	Thymine (only in sections 1.10 and 3.1)
V	Volts
VFA	Volatile Fatty Acids
W	Watt
WW	Wastewater
X-Gal	5-Bromo-4-Chloro-3-Indolyl- B-D-Galactopyranoside
Ω	Ohm

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Curriculum Vitae

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EDUCATION

2011-Present **Ph.D. Student in Biotechnology**. Universitat Autònoma de Barcelona, Spain with PhD thesis title: "A microbiological approach to improve the performance of single-chamber bioelectrochemical systems"

2009-2011 **Degree in Pharmaceutical, Veterinary and Medical Biotechnologies (Master level).** Università Magna Græcia di Catanzaro. Experimental graduation thesis: "FISH" Fluorescence In Situ Hibridization for the Study of Microbial Population of a Percolating Biofilter for Treatment of H_2S Gaseous Phase". Internship (14 months) in the Chemical Engineer Department of Universitat Autònoma de Barcelona

2004-2008 **Degree in Biotechnology.** Università Magna Græcia di Catanzaro. Experimental graduation thesis: "Use of RAMAN Technology Applied to Nanostructured Supports for the Evaluation of Puntiform Mutations". Internship (10 months) in the Nanotechnology Laboratory/Clean Room Bionem Group, Università Magna Græcia di Catanzaro.

WORK EXPERIENCE

2011-Present **Universitat Autònoma de Barcelona**. Researcher student in the Department of Chemical Engineering.

SCHOLARSHIPS

2013 **Stage Scholarships in Arizona F.P.I.** Swette Center of Biodesign Institute of Arizona State University in Tempe, USA

2011-Present **Pre-doctoral Scholarships in Spain F.P.I.** Universitat Autònoma de Barcelona, Spain

2010-2011 **European Union program LLP Erasmus Placement** (6 months). Department of Chemical Engineering of the Universitat Autònoma de Barcelona, Spain

2010 **European Union program LLP Erasmus** (8 months). Department of Chemical Engineering of the Universitat Autònoma de Barcelona, Spain

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TECHNICAL SKILLS

- **Growth of pure and mixed bacteria cultures and reactors**
- **Cloning (DNA Ligation) and electroporation bacterial transformation**
- **Fluorescence in-Situ Hybridization** for the study of microbial population
- **Real Time Polymerase Chain Reaction** for absolute quantification
- **Use of Scanning Electron Microscopy (SEM)** for the study of microbial biofilms
- **Use of RAMAN Technology** applied to nanostructured supports
- **Use of Confocal Microscopy**
- **High Throughput 16S Pyrosequencing and bioinformatics analysis**
- Assembly and maintenance of **MFCs** and of **MECs**
- **Use of potentiostat**
- **Electricity and hydrogen production by electrochemical systems**
- **Electricity and hydrogen production from dairy wastewater and whey**
- **Use of gas and ionic chromatography**

LANGUAGE SKILLS

Italian		Mother Tongue
Spanish	**Oral:**	Excellent
	Written:	Excellent
English	**Oral:**	Advanced
	Written:	Advanced

PUBBLICATIONS

JOURNAL ARTICLES:

L. Rago, J. Guerrero, J. A. Baeza, A. Guisasola (2015), 2-Bromoethanesulfonate degradation in bioelectrochemical systems. Bioelectrochemistry, 105, 44–49. doi:10.1016/j.bioelechem.2015.05.001

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E. Ribot-Llobet, N. Montpart, Y. Ruiz-Franco, L. Rago, J. Lafuente, J.A. Baeza, A. Guisasola, (2013), Obtaining microbial communities with exoelectrogenic activity from anaerobic sludge using a simplified procedure. Journal of Chemical Technology and Biotechnology. doi: 10.1002/jctb.4252

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L. Rago, J. A. Baeza, A. Guisasola (submitted - Water Science and Technology) Performance of microbial electrolysis cells with bioanodes grown at different external resistances

L. Rago, J. A. Baeza, A. Guisasola (in preparation) High performance of alkaline bioelectrochemical hydrogen production

L. Rago, J. A. Baeza, A. Guisasola (in preparation) Bioelectrochemical hydrogen production with cheese whey as sole substrate

R.A. Yoho, S.C. Popat, L. Rago, A. Guisasola, C.I. Torres (in preparation) Anode biofilms of *Geoalkalibacter ferrihydriticus* exhibit electrochemical signatures of multiple electron transport pathways

CONGRESS RECORDS:

Y. Ruiz, L. Rago, P. Cortés, J.A. Baeza, A. Guisasola. , Identifying hydrogen scavengers in a single-chamber membrane-less microbial electrolysis cells. Organization: The 4th International Microbial Fuel Cells Conference in Cairns, Australia. 2013

N. Montpart, E. Ribot, V.K. Garlapati, L. Rago, J.A. Baeza, A. Guisasola , Systematic development of anodic syntrophic consortia for bioelectrochemical hydrogen production from a wider range of carbon source. Organization: The 4th International Microbial Fuel Cells Conference in Cairns, Australia. 2013.

L. Rago, N. Montpart, J.A. Baeza, J.A. and A. Guisasola. The different roles of the cathodic biofilm in air-cathode Microbial Fuel Cells. Organization: The 2nd European meeting of the International Society for Microbial Electrochemistry and Technologies (ISMET**)** Alcalá de Henares (Madrid), Spain. 2014.

L. Rago, M. Badia, J.A. Baeza, A. Guisasola. Extending the applicability range for bioelectrochemical systems: prospects for alkaline exoelectrogenesis. Organization: The 5th International Meeting on Microbial Electrochemistry and Technologies (ISMET). Arizona State University, Tempe (USA), October, 2015.

L. Rago, J. Guerrero, Y. Ruiz, J.A. Baeza, A. Guisasola. Experiences with real cheese-whey wastewater in bioelectrochemical hydrogen production: from lab to pilot scale. Organization: The 5th International Meeting on Microbial Electrochemistry and Technologies (ISMET). Arizona State University, Tempe (USA), October, 2015.